FILE 'HOME' ENTERED AT 15:38:38 ON 24 MAY 2001

=> file medline biosis biotechno

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	1.95	1.95

FILE 'MEDLINE' ENTERED AT 15:46:23 ON 24 MAY 2001

FILE 'BIOSIS' ENTERED AT 15:46:23 ON 24 MAY 2001

FILE 'BIOTECHNO' ENTERED AT 15:46:23 ON 24 MAY 2001

=> s subunit of PKA

L1 588 SUBUNIT OF PKA

=> s l1 and cre

L2 26 L1 AND CRE

=> dup rem l2

PROCESSING COMPLETED FOR L2
L3 11 DUP REM L2 (15 DUPLICATES REMOVED)

=> d ibib abs 1-11

L3 ANSWER 1 OF 11 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 1998283992 MEDLINE
DOCUMENT NUMBER: 98283992 PubMed ID: 9618473
TITLE: The RIIbeta regulatory subunit of protein kinase A binds to cAMP response element: an alternative cAMP signaling pathway.
AUTHOR: Srivastava R K; Lee Y N; Noguchi K; Park Y G; Ellis M J; Jeong J S; Kim S N; Cho-Chung Y S
CORPORATE SOURCE: Cellular Biochemistry Section, Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Building 10, Room 5B05, Bethesda, MD 20892-1750, USA.
SOURCE: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 Jun 9) 95 (12) 6687-92. Journal code: PV3; 7505876. ISSN: 0027-8424.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199807
ENTRY DATE: Entered STN: 19980716
Last Updated on STN: 19990129
Entered Medline: 19980709

AB cAMP, through the activation of cAMP-dependent protein kinase (PKA), is involved in transcriptional regulation. In eukaryotic cells, cAMP is not considered to alter the binding affinity of CREB/ATF to cAMP-responsive element (**CRE**) but to induce serine phosphorylation and consequent increase in transcriptional activity. In contrast, in prokaryotic cells, cAMP enhances the DNA binding of the catabolite

repressor protein to regulate the transcription of several operons. The structural similarity of the cAMP binding sites in catabolite repressor protein and regulatory **subunit** of **PKA** type II (RII) suggested the possibility of a similar role for RII in eukaryotic gene regulation. Herein we report that RIIbeta **subunit** of **PKA** is a transcription factor capable of interacting physically and functionally with a **CRE**. In contrast to CREB/ATF, the binding of RIIbeta to a **CRE** was enhanced by cAMP, and in addition, RIIbeta exhibited transcriptional activity as a Gal4-RIIbeta fusion protein. These experiments identify RIIbeta as a component of an alternative pathway for regulation of **CRE**-directed transcription in eukaryotic cells.

L3 ANSWER 2 OF 11 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 97183729 MEDLINE
DOCUMENT NUMBER: 97183729 PubMed ID: 9031689
TITLE: Transient **CRE**- and kappa B site-binding is cross-regulated by cAMP-dependent protein kinase and a protein phosphatase in mouse splenocytes.
AUTHOR: Koh W S; Jeon Y J; Herring A C; Kaminski N E
CORPORATE SOURCE: Department of Pharmacology and Toxicology, Michigan State University, East Lansing 48824, USA.
CONTRACT NUMBER: DA07908 (NIDA)
DA09171 (NIDA)
SOURCE: LIFE SCIENCES, (1997) 60 (6) 425-32.
Journal code: L62; 0375521. ISSN: 0024-3205.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199703
ENTRY DATE: Entered STN: 19970313
Last Updated on STN: 19970313
Entered Medline: 19970305

AB Cyclic AMP regulates a variety of cellular responses through activation of cAMP-dependent protein kinase (PKA). The catalytic **subunit** of **PKA**, in turn, activate cAMP responsive element (**CRE**) and nuclear factor-kappa B (NF-kappa B) binding proteins. In this study, we demonstrated that binding activity to both **CRE** and kappa B sites in nuclear extracts from spleen cells is modulated by PKA in a time-dependent manner. Electrophoretic mobility shift assays showed that binding by transcription factors to either the **CRE** or kappa B motif was rapidly up-regulated by cAMP, with maximum binding detected at 30 min in response to forskolin stimulation of splenocytes. This was followed by a steady decline in **CRE** and kappa B thereafter reaching basal levels by 2 hr. This up-regulation in **CRE** and kappa B binding was closely associated with an enhancement of PKA activity which was maximum at 30 min following forskolin stimulation. However, unlike the binding of regulatory factors to **CRE** and kappa B motifs which was very transient, peak PKA activity was sustained for 2 hr. Interestingly, okadaic acid, a protein phosphatase inhibitor, prevented the decline in protein binding to **CRE** and kappa B motifs 2 hr following forskolin stimulation and actually produced a slight increase at 30 min. These data suggest that binding by transcription factors to **CRE** and kappa B sites are up-regulated concomitantly with PKA activation but subsequently down-regulated by a protein phosphatase.

L3 ANSWER 3 OF 11 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 97191700 MEDLINE
DOCUMENT NUMBER: 97191700 PubMed ID: 9039646
TITLE: Transcriptional regulation of neurofilament expression by protein kinase A.
AUTHOR: White L A; Reeben M; Saarma M; Whittemore S R
CORPORATE SOURCE: Miami Project, University of Miami School of Medicine, Florida, USA.

CONTRACT NUMBER: [redacted]6887 (NINDS)
SOURCE: [redacted]RNAL OF NEUROSCIENCE RESEARCH, (1997 Feb 1) 47 (3) 242-52.
Journal code: KAC; 7600111. ISSN: 0360-4012.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199704
ENTRY DATE: Entered STN: 19970507
Last Updated on STN: 19970507
Entered Medline: 19970428

AB RN46A cells, a conditionally immortalized neuronal cell line derived from E12 rat medullary raphe nucleus, upregulate low M(r) (68 kDa, neurofilament [NF]-L) and medium M(r) (160 kDa, NF-M) neurofilament protein expression upon activation of protein kinase A (PKA). To examine possible transcriptional regulation of neurofilament protein expression by PKA, two cell lines were used; RN46A cells and C alpha EV6 cells, a cell line derived from RN46A cells that stably expresses the catalytic **subunit** of **PKA** under the control of the metallothionein promoter. Treatment of RN46A cells with dbcAMP resulted in an increase in the steady-state levels of both NF-L and NF-M, but not high M(r) (200 kDa, NF-H) neurofilament mRNA. These increases were both time and dose dependent and were sensitive to treatment with the protein synthesis inhibitor cycloheximide. In C alpha EV6 cells, activation of PKA by 80 microM ZnSO4 upregulated the expression of C alpha mRNA with maximal levels reached 8 hr post-treatment and maintained at 24 hr. Reporter gene assays in C alpha EV6 cells following transfection with increasing lengths of the NF-L promoter demonstrated that both a putative Sp1-like and a cAMP response (**CRE**), but not a NGFI-A, element were likely involved in PKA-dependent activation of the NF-L promoter. Electrophoretic mobility shift assays confirmed these results but showed that the nuclear proteins induced by PKA which bound to the NF-L promoter Sp1-like sequence were not Sp1. Collectively, these data suggest that constitutively expressed Sp1 may be involved in basal NF-L promoter activity, and newly synthesized, PKA-dependent nuclear proteins may synergistically activate the rat NF-L promoter.

L3 ANSWER 4 OF 11 BIOTECHNO COPYRIGHT 2001 Elsevier Science B.V.
ACCESSION NUMBER: 1996:26297151 BIOTECHNO
TITLE: Effects of 8-chloroadenosine on murine hepatoma H.sub.2.sub.2 cells
AUTHOR: Zhan J.-H.; Fang J.-C.; Shi Y.-J.; Peng J.; Wang D.-S.; Liang Y.-Y.
CORPORATE SOURCE: Beijing Inst. for Cancer Research,Beijing 100034, China.
SOURCE: Chinese Pharmacological Bulletin, (1996), 12/1 (71-73)
CODEN: ZYTOE8 ISSN: 1001-1978
DOCUMENT TYPE: Journal; Article
COUNTRY: China
LANGUAGE: Chinese
SUMMARY LANGUAGE: Chinese; English

AN 1996:26297151 BIOTECHNO
AB 8-Chloroadenosine showed marked activity against murine hepatoma H.sub.2.sub.2 solid tumor, at 100 mg.midldot.kg.sup.-.sup.1.midldot.d.sup.-.sup.1 x 7 d. The inhibition rates of H.sub.2.sub.2 were 71.3 .+-. 13.3% (P<0.01) and 66.1 .+-. 4.46% (P<0.01) by ip. and iv. administration respectively. 8-Cl-Ado increased intercellular cAMP concentration but decreased diacylglycerol amount in H.sub.2.sub.2 ascites cells. Photoaffinity labeling/SDS-polyacrylamide gel

electrophoresis assay indicated that 8-Cl-Ado greatly decreased R I (regulatory **subunit of PKA**) in the cytosol of H.sub.2.sub.2 cells. Gel retardation analysis demonstrated the enhancement of **CRE**-binding activity after treatment for 24 hours with 8-Cl-Ado. Using in situ hybridization, 8-Cl-Ado induced antioncogene p 53 expression.

L3 ANSWER 5 OF 11 MEDLINE DUPLICATE 4
ACCESSION NUMBER: 95098018 MEDLINE
DOCUMENT NUMBER: 95098018 PubMed ID: 7799950
TITLE: Impaired cyclic AMP-dependent phosphorylation renders CREB a repressor of C/EBP-induced transcription of the somatostatin gene in an insulinoma cell line.
AUTHOR: Vallejo M; Gosse M E; Beckman W; Habener J F
CORPORATE SOURCE: Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Howard Hughes Medical Institute, Boston 02114.
CONTRACT NUMBER: DK30457 (NIDDK)
DK30834 (NIDDK)
SOURCE: MOLECULAR AND CELLULAR BIOLOGY, (1995 Jan) 15 (1) 415-24.

Journal code: NGY; 8109087. ISSN: 0270-7306.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199501
ENTRY DATE: Entered STN: 19950215
Last Updated on STN: 19970203
Entered Medline: 19950124

AB Transcription factor CREB regulates cyclic AMP (cAMP)-dependent gene expression by binding to and activating transcription from cAMP response elements (CREs) in the promoters of target genes. The transcriptional transactivation functions of CREB are activated by its phosphorylation by cAMP-dependent protein kinase A (PKA). In studies of many different phenotypically distinct cells, the **CRE** of the somatostatin gene promoter is a prototype of a highly cAMP-responsive element regulated by CREB. We now report on a somatostatin-producing rat insulinoma cell line, RIN-1027-B2, in which transcription from the somatostatin gene promoter is paradoxically repressed by CREB. We find that CREB fails to transactivate a **CRE**-containing somatostatin-chloramphenicol acetyltransferase reporter even when coexpressed with the catalytic **subunit** of **PKA**. CAAT box/enhancer-binding protein beta (C/EBP beta) and C/EBP-related activating transcription factor bind to the **CRE** in the promoter of the somatostatin gene and transactivate transcription. CREB binds competitively with C/EBP beta to the somatostatin **CRE** in vitro and represses C/EBP beta-induced transcription of the **CRE** -containing somatostatin-chloramphenicol acetyltransferase reporter. The lack of CREB-mediated transcriptional stimulation is due to the presence of a heat-stable inhibitor of PKA that prevents activation of PKA and subsequent CREB phosphorylation in the nucleus. These findings indicate that dephosphorylated CREB is a negative regulator of C/EBP-activated transcription of the somatostatin gene promoter in RIN-1027-B2 cells.

L3 ANSWER 6 OF 11 BIOTECHNO
ACCESSION NUMBER: 1995:25146599 BIOTECHNO
TITLE: Glucagon receptors: From genetic structure and expression to effector coupling and biological responses
AUTHOR: Christophe J.
CORPORATE SOURCE: Department of Experimental Surgery, Medical School, Universite Libre, 40, Avenue J. Wybran,B-1070 Brussels, Belgium.
SOURCE: Biochimica et Biophysica Acta - Reviews on Biomembranes, (1995), 1241/1 (45-57)
CODEN: RVBMA3 ISSN: 0304-4157
DOCUMENT TYPE: Journal; General Review
COUNTRY: Netherlands

LANGUAGE: English
SUMMARY LANGUAGE: English
AN 1995:25146599 BIOTECHNO
AB The 1455 bp rat hepatic glucagon receptor ORF encodes 485 amino acids for a G-protein coupled protein with 7 transmembrane (TM) segments. The deduced amino acid sequence shows 42% identity with the rat GLP-1 receptor. Transfection of this receptor into COSGs1 cells allows selective glucagon binding and adenylyl cyclase stimulation. It now appears that the rat glucagon receptor gene contains 12 exons, 7 of which code for the TM domain. The gene is transcribed into several pre-mRNAs, variously shortened at the 5' end. One mature intronless mRNA, after the splicing out of the 11 introns, is translated into the functional glucagon receptor. We detected by PCR the apparent expression of the same glucagon receptor in rat liver, heart, islets (.beta. cells?), stomach, kidney and adipocytes, suggesting that one gene allows the expression of only one type of glucagon receptor product, in terms of amino acid sequence. To further analyze the structure-activity relationship of this important yet strictly localized receptor four lines of research are now obvious: (1) To examine the bearing of posttranslational processing by glycosylation, phosphorylation and palmitoylation. (2) The DNA encoding the glucagon receptor being now stably transfected in CHO cells, this will hopefully allow to identify, at the atomic level, the interaction of glucagon with the receptor-effector complex. Such a transfected receptor, well expressed and coupled to adenylate cyclase, can indeed serve as reference when testing plasmids with partial deletions or point mutations (to alter charges), and chimeric constructions (where a fragment of the glucagon receptor is substituted by the corresponding fragment of a parent receptor, e.g., the tGLP-1 receptor). Mutagenesis of extracellular Asn and Cys residues will reveal the importance of glycosylation and disulfide bridges as prerequisites for receptor function. This evaluation will probably require the use of specific antibodies to see whether a given mutation is not responsible for a mere three-dimensional delocalization and general instability (inactivity) of the receptor synthesized by CHO cells. The binding and functional data collected will not only reveal specific roles for each extra- and intracellular domain of the receptor, they will also indicate how the side chains of residues His1, Gly4, Asp9, Lys12 and Ser16 in glucagon are sterically involved in effector coupling, giving clues in our search for pharmacologically valid analogs. (3) Within the first 104 bp of the 5'-flanking region .cents.91!, the TGAGCTCA sequence starting at position - 96 is similar to the consensus sequence TGACGTCA for **CRE**, and the ACCCAGGC sequence starting at position -50 could be related to the consensus sequence CCCCAGGC for factor AP-2 (that responds to both PKC and PKA). It is important to evaluate the regulation of receptor mRNA transcription with a full characterization (primary DNA sequence, placement, spacing, multiplicity) of regions of promoter sites that contain cis-acting enhancers, such as cAMP-responsive element **CRE** and tissue-specific elements. These elements could be regulated positively or negatively by trans-acting transcription factors and cofactors reacting to either cAMP (via protein-protein recognition with the C **subunit of PKA**), phosphorylation, hormones (corticosterone, insulin) or nutrients (glucose, polyunsaturated fatty acids). Expression assays and transgenic mouse technology could be used to identify these gene regulatory elements and the cell-specific transcription factors that control the limited tissue distribution of this receptor. (4) Appropriate primers will allow a quantitative PCR assay of mRNA levels for glucagon receptors, under various pathological

conditions. For instance, in congenital obesity and hypertension in rodents, a change in receptor number in the tissues may reflect alterations in transcription rate and/or mRNA stability. Besides, a precise cellular localization of the receptor mRNA, by in situ hybridization procedures, could delineate whether .beta. and .delta. cells are capable of expressing glucagon receptors and of modulating this synthesis, in response to glucagon secreted by .alpha. cells in the same islets.

L3 ANSWER 7 OF 11 MEDLINE DUPLICATE 5
ACCESSION NUMBER: 94237840 MEDLINE
DOCUMENT NUMBER: 94237840 PubMed ID: 8182041
TITLE: Inhibition by insulin of protein kinase A-induced transcription of the phosphoenolpyruvate carboxykinase gene. Mediation by the activation domain of cAMP response element-binding protein (CREB) and factors bound to the TATA box.
AUTHOR: Quinn P G
CORPORATE SOURCE: Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey 17033.
CONTRACT NUMBER: DK43871 (NIDDK)
SOURCE: JOURNAL OF BIOLOGICAL CHEMISTRY, (1994 May 20) 269 (20) 14375-8.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199406
ENTRY DATE: Entered STN: 19940621
Last Updated on STN: 19980206
Entered Medline: 19940616

AB The minimal promoter/transcription factor requirements for induction of phosphoenolpyruvate carboxykinase (PEPCK) transcription by cAMP-activated protein kinase A (PKA) and inhibition of this induction by insulin were investigated. H4 hepatoma cells were treated with or without insulin following cotransfection with chloramphenicol acetyltransferase reporter genes and expression vectors coding for the cAMP response element-binding protein (CREB) activation domain fused to the GAL4 DNA binding domain (CRG) and the catalytic **subunit** of **PKA**. Mutation of the PEPCK **CRE** to a GAL4 binding site (G4-PEPCK) within the fully responsive PEPCK promoter (-600/+69) made induction by PKA dependent upon cotransfection of CRG and this induction by CRG+PKA was inhibited by insulin. Mutation of the insulin regulatory sequence (delta IRS-G4-PEPCK) did not prevent induction by cAMP or inhibition by insulin. Fusion of GAL4 binding sites to the PEPCK TATA region (-40/+1, G4-PT) allowed induction by CRG+PKA and inhibition by insulin. However, inhibition by insulin was not observed when the CREB activation domain in CRG was replaced with the activation domain of VP16 (G4-VP16) or when the PEPCK TATA region was replaced with TATA regions from other genes. Our results indicate that the minimal requirements for induction of PEPCK by PKA and inhibition by insulin include: 1) the CREB activation domain, 2) the PEPCK TATA sequence, and 3) insulin-responsive hepatoma cells. These data suggest that specific factors interacting with both the PEPCK TATA region and the CREB activation domain are required for insulin inhibition of PKA-induced transcription.

L3 ANSWER 8 OF 11 MEDLINE DUPLICATE 6
ACCESSION NUMBER: 95054429 MEDLINE
DOCUMENT NUMBER: 95054429 PubMed ID: 7525897
TITLE: The cAMP-dependent protein kinase regulates transcription of the dopamine beta-hydroxylase gene.
AUTHOR: Kim K S; Ishiguro H; Tinti C; Wagner J; Joh T H
CORPORATE SOURCE: Department of Neurology and Neuroscience, Cornell University Medical College, W. M. Burke Medical Research

Institute, White Plains, New York 10605.
CONTRACT NUMBER: [redacted]8866 (NIMH)
SOURCE: JOURNAL OF NEUROSCIENCE, (1994 Nov) 14 (11 Pt 2) 7200-7.
Journal code: JDF; 8102140. ISSN: 0270-6474.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199412
ENTRY DATE: Entered STN: 19950110
Last Updated on STN: 19970203
Entered Medline: 19941202

AB Dopamine beta-hydroxylase (DBH) catalyzes the conversion of dopamine to norepinephrine, and is expressed specifically in neurons and neuroendocrine cells that release norepinephrine and epinephrine. In the present study, we used DBH-expressing human neuroblastoma SK-N-BE(2)C and rat pheochromocytoma (PC12) cell lines to investigate the role of cAMP-dependent protein kinase (PKA) in transcriptional regulation of the DBH gene. Coexpression of the catalytic **subunit** of **PKA** (PKAc) robustly stimulated the transcriptional activity of the DBH gene in a dose-dependent manner. Conversely, coexpression of a specific inhibitor of PKA abrogated forskolin- and cAMP-mediated but not phorbol ester-mediated transcriptional induction of DBH. Deletion of the cAMP response element (**CRE**) dramatically reduced the stimulatory effect of PKA, indicating that the **CRE** mediates the induction of DBH by PKA. In DBH-nonexpressing HeLa and C6 glioma cell lines, coexpression of PKAc changed the transcriptional activity of the DBH promoter to a minimal degree, indicating that basal and PKA-mediated transcription of the DBH gene occur in a cell type-specific manner. Finally, both basal and cAMP-stimulated transcription of the DBH gene are diminished in three PKA-deficient PC12 cell lines, compared to wild-type cells. Based on these data, we conclude that PKA, via the **CRE**, plays an important role in basal and cAMP-inducible transcription, but is not required for phorbol ester-mediated induction, of the DBH gene in noradrenergic cells.(ABSTRACT TRUNCATED AT 250 WORDS)

L3 ANSWER 9 OF 11 MEDLINE DUPLICATE 7
ACCESSION NUMBER: 95089772 MEDLINE
DOCUMENT NUMBER: 95089772 PubMed ID: 7997232
TITLE: Thyroid-specific expression and cyclic adenosine 3',5'-monophosphate autoregulation of the thyrotropin receptor gene involves thyroid transcription factor-1.
AUTHOR: Shimura H; Okajima F; Ikuyama S; Shimura Y; Kimura S; Saji M; Kohn L D
CORPORATE SOURCE: Section on Cell Regulation, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892.
SOURCE: MOLECULAR ENDOCRINOLOGY, (1994 Aug) 8 (8) 1049-69.
Journal code: NGZ; 8801431. ISSN: 0888-8809.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199501
ENTRY DATE: Entered STN: 19950126
Last Updated on STN: 19970203
Entered Medline: 19950119

AB The chimeric chloramphenicol acetyltransferase (CAT) construct, pTRCAT5'-199, containing the TSH receptor (TSHR) minimal promoter, -199 to -39 base pairs (bp), exhibits the thyroid specificity and TSH/cAMP autoregulation evident in TSHR gene expression. The present report shows that a cis-acting element between -189 and -175 bp, which binds thyroid transcription factor-1 (TTF-1), is involved in both activities. The 22 bp between -199 and -178 contains a positive element important for expression of the TSHR minimal promoter in rat FRTL-5 thyroid cells. DNAase I footprinting shows that extracts from functioning FRTL-5, but not

non-functioning FRT thyroid or Buffalo rat liver BRL) cells, protect a region between -199 and -175 bp. The protection is duplicated by TTF-1, and the protected element has only a two-base mismatch from the consensus TTF-1 element identified in the thyroglobulin (TG) and thyroid peroxidase minimal promoters. Gel mobility shift analyses reveal that FRTL-5 thyroid cell nuclear extracts form a specific protein/DNA complex with this region, which is prevented by the TTF-1 binding element from the TG promoter; FRT and BRL cell nuclear extracts do not have TTF-1 and do not form this complex. A role for the TSHR/TTF-1 binding element in thyroid-specific expression of the TSHR gene is evidenced as follows. Overexpression of TTF-1 in FRT or BRL cells, which have no TTF-1, increased the activity of pTRCAT5'-199, but not pTRCAT5'-177, which has no TTF-1 binding element. A nonsense mutation of the TTF-1 binding element eliminated TTF-1-induced activation of TSHR promoter activity in FRT or BRL cells and reduced TSHR promoter activity in FRTL-5 thyroid cells. In contrast, mutation of this element to the TTF-1 consensus sequence of the TG or thyroid peroxidase promoter had no significant influence on TSHR promoter activity. The activity of the TSHR/TTF-1 binding element requires a functioning cAMP response element (**CRE**). Thus, TTF-1 activity is lost when the **CRE** site is mutated to a nonfunctional, nonpalindromic sequence; it is, in contrast, maximized when **CRE** activity is maximized by its mutation to a consensus AP1 element. TTF-1 phosphorylation is important for binding and activity. Thus, binding of TTF-1 to the TSHR/TTF-1 element is phosphatase-sensitive and is increased by treating nuclear extracts with the catalytic subunit of protein kinase A. Overexpression of the catalytic **subunit** of **PKA** enhances TTF-1-increased activity of the TSHR minimal promoter.(ABSTRACT TRUNCATED AT 400 WORDS)

L3 ANSWER 10 OF 11 MEDLINE DUPLICATE 8

ACCESSION NUMBER: 94328135 MEDLINE
DOCUMENT NUMBER: 94328135 PubMed ID: 7914223
TITLE: Cyclic AMP-dependent protein kinase regulates basal and cyclic AMP-stimulated but not phorbol ester-stimulated transcription of the tyrosine hydroxylase gene.
AUTHOR: Kim K S; Tinti C; Song B; Cubells J F; Joh T H
CORPORATE SOURCE: Laboratory of Molecular Neurobiology, W. M. Burke Medical Research Institute, Cornell University Medical College, White Plains, New York 10605.
CONTRACT NUMBER: MH 24285 (NIMH)
MH 48866 (NIMH)
SOURCE: JOURNAL OF NEUROCHEMISTRY, (1994 Sep) 63 (3) 834-42.
Journal code: JAV; 2985190R. ISSN: 0022-3042.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199409
ENTRY DATE: Entered STN: 19940914
Last Updated on STN: 19980206
Entered Medline: 19940908

AB To define the precise role of cyclic AMP (cAMP)-dependent protein kinase (PKA) in transcriptional regulation of the tyrosine hydroxylase (TH) gene, we performed transient cotransfection analyses of a reporter construct containing the upstream 2,400 bp sequence of the rat TH gene with expression plasmids encoding a heat-stable specific inhibitor of PKA (PKI), a mutant regulatory **subunit** of **PKA**, or the catalytic **subunit** of **PKA**. Inhibition of PKA activity by expression of either PKI or mutant regulatory subunit blocked cAMP-stimulated induction and reduced basal transcription of the TH-reporter construct. Expression of the catalytic **subunit** of **PKA** induced the expression of the TH-reporter construct up to 50-fold in a dose-dependent manner. Primer extension analysis confirmed that PKA-mediated induction of TH-reporter expression occurred at the correct transcription initiation site. Expression of PKI did not affect induction following phorbol ester treatment, suggesting that PKA and

protein kinase [redacted]PKC) induce TH transcription by [redacted]ndependent mechanisms. Finally, a doub[redacted]mutation within the cAMP response element (**CRE**) of TH2400-CAT diminished its basal and forskolin-stimulated transcription to the level of the promoterless plasmid, pBLCAT3, but did not alter the induction following treatment with phorbol ester, indicating that the **CRE** is not required for PKC-mediated transcriptional induction. Our results indicate that PKA, via the **CRE**, plays a crucial role for basal and cAMP-inducible transcription of the TH gene.

L3 ANSWER 11 OF 11 MEDLINE DUPLICATE 9
ACCESSION NUMBER: 93124059 MEDLINE
DOCUMENT NUMBER: 93124059 PubMed ID: 8380441
TITLE: Characterization and genetic analysis of functional corticotropin-releasing hormone receptors in primary cerebellar cultures.
AUTHOR: Barthel F; Loeffler J P
CORPORATE SOURCE: Laboratoire de Neurophysiologie et de Neurobiologie des Systemes Endocrines, URA 1446 du CNRS, Universite Louis Pasteur, Strasbourg, France.
SOURCE: JOURNAL OF NEUROCHEMISTRY, (1993 Feb) 60 (2) 696-703. Journal code: JAV; 2985190R. ISSN: 0022-3042.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199302
ENTRY DATE: Entered STN: 19930226
Last Updated on STN: 19980206
Entered Medline: 19930209

AB We demonstrate that granular cerebellar neurons express functional corticotropin-releasing hormone (CRH) receptors. Activation of these receptors with CRH receptor agonists leads to a dose-dependent increase in cyclic AMP (cAMP) levels with an apparent EC50 close to 10(-9) M. Using the c-fos protooncogene as a system to evaluate genomic effects of CRH, we show that activation of CRH receptors regulates gene expression at the transcriptional level. CRH rapidly induced c-fos mRNA accumulation. Genetic studies, using chimera genes containing human c-fos promoter sequences coupled to a chloramphenicol acetyltransferase (CAT) reporter gene, confirmed and extended this observation. When protein kinase A (PKA) was specifically inactivated by gene transfer of a mutated regulatory **subunit** of **PKA** lacking cAMP binding sites, CRH-stimulated c-fos transcription was suppressed but the increase in cAMP level was not affected, indicating a key role of PKA in mediating CRH-stimulated transcription. As CRH clearly modulates gene expression via the cAMP pathway, we analyzed the genomic effect of this neurohormone on a deleted c-fos-CAT construct containing only the cAMP-responsive element (**CRE**) and on a heterologous promoter construct bearing the minimal palindromic consensus **CRE** (core sequence TGACGTCA). These minimal cAMP-responsive genes are induced by CRH. These inductions are dependent on functional PKA. Taken together, our results demonstrate the presence of functional CRH receptors in primary cerebellar cultures. Activation of these receptors stimulates gene expression via the cAMP/PKA pathway and the transacting factor CREB (cAMP-responsive element binding protein).

=> d his

(FILE 'HOME' ENTERED AT 15:38:38 ON 24 MAY 2001)

FILE 'MEDLINE, BIOSIS, BIOTECHNO' ENTERED AT 15:46:23 ON 24 MAY 2001

L1 588 S SUB[redacted]T OF PKA
L2 26 S L1 [redacted] CRE
L3 11 DUP REM L2 (15 DUPLICATES REMOVED)

=> s cre-creb

L4 19 CRE-CREB

=> dup rem l4

PROCESSING COMPLETED FOR L4
L5 10 DUP REM L4 (9 DUPLICATES REMOVED)

=> d ibib abs 1-10

L5 ANSWER 1 OF 10 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 2000412218 MEDLINE
DOCUMENT NUMBER: 20314398 PubMed ID: 10854708
TITLE: Early induction of the orphan nuclear receptor NOR-1 during
cell death of the human breast cancer cell line MCF-7.
AUTHOR: Ohkubo T; Ohkura N; Maruyama K; Sasaki K; Nagasaki K; Hanzawa H; Tsukada T; Yamaguchi K
CORPORATE SOURCE: Growth Factor Division, National Cancer Center Research Institute, Tokyo, Japan.
SOURCE: MOLECULAR AND CELLULAR ENDOCRINOLOGY, (2000 Apr 25) 162 (1-2) 151-6.
Journal code: E69; 7500844. ISSN: 0303-7207.
PUB. COUNTRY: Ireland
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200008
ENTRY DATE: Entered STN: 20000907
Last Updated on STN: 20000907
Entered Medline: 20000828

AB The neuron-derived orphan receptor (NOR-1) is a member of the NGFI-B subfamily within the nuclear receptor superfamily. In T-cell apoptosis, where NGFI-B plays an essential role, a functional redundancy between NGFI-B and NOR-1 has been demonstrated. Here, we examined the regulation and expression of the NOR-1 gene during cell death induced by a calcium ionophore A23187 in the human breast cancer cell line MCF-7. A23187 caused a transient increase in NOR-1 mRNA levels within 6 h after treatment. To delineate the sequences required for the transitional response to A23187, a series of promoter deletion mutants were constructed. From the transient transfection experiments, the element responsive to A23187 was identified between -94 and -42 base pairs upstream from the transcription initiation site. This 53-base pairs region contains three copies of the cAMP response element (CRE). Furthermore, phosphorylation of the CRE-binding protein (CREB), which affects the transcription of the CRE dependent-genes, was detected 30 min after A23187 stimulation. Our findings are consistent with NOR-1 involvement in A23187-induced cell death via the **CRE-CREB** signaling pathway.

L5 ANSWER 2 OF 10 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 2000:216226 BIOSIS
DOCUMENT NUMBER: PREV200000216226
TITLE: Interference of Pur alpha with a double stranded somatostatin CRE (ds-somaCRE)/CREB pathway.
AUTHOR(S): Kuo, Che-Hui (1); Nishikawa, Etsuko (1); Sadakata, Tetsushi (1); Kumamaru, Emi (1); Niu, San-Yong (1); Miki, Naomasa (1)
CORPORATE SOURCE: (1) Department of Pharmacology, Osaka University Medical

ool, Osaka, 565-0871 Japan
SOURCE: anese Journal of Pharmacology, (2000) Vol. 82, No. Suppl. 1, pp. 59P.
Meeting Info.: 73rd Annual Meeting of the Japanese Pharmacological Society. Yokohama, Japan March 23-25, 2000
ISSN: 0021-5198.
DOCUMENT TYPE: Conference
LANGUAGE: English
SUMMARY LANGUAGE: English

L5 ANSWER 3 OF 10 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 2001:60444 BIOSIS
DOCUMENT NUMBER: PREV200100060444
TITLE: Inhibition of CRE-mediated gene expression by CREB within the context of an apoptotic stimulus.
AUTHOR(S): Francis, J. S. (1); During, M. J.
CORPORATE SOURCE: (1) Thomas Jefferson Univ, Philadelphia, PA USA
SOURCE: Society for Neuroscience Abstracts, (2000) Vol. 26, No. 1-2, pp. Abstract No.-49.16. print.
Meeting Info.: 30th Annual Meeting of the Society of Neuroscience New Orleans, LA, USA November 04-09, 2000 Society for Neuroscience
. ISSN: 0190-5295.
DOCUMENT TYPE: Conference
LANGUAGE: English
SUMMARY LANGUAGE: English
AB Activation of the cAMP response element binding protein (CREB) is a feature of the cellular response to insults that are associated with apoptosis. CREB is thought to activate target gene expression primarily through an interaction with the cAMP response element (CRE) found within the promoter of these genes. An in vitro model of stress-activated cellular signaling was used to investigate the activity of CRE-mediated transcriptional activity within the context of an apoptotic insult. Mouse C17.2 neural precursor cells were transfected with a CRE-containing luciferase reporter cassette prior to exposure to okadiac acid, in order to investigate the role of cAMP-mediated transcriptional activation within an apoptotic context. Exposure of cells to okadiac acid resulted in a 25-fold induction of luciferase expression that was detectable as early as 15 minutes after exposure, and maximal after 6 hours. Cotransfection of a **CRE-CREB** cassette significantly reduced okadiac acid-associated induction of luciferase expression. This apparent inhibition by **CRE-CREB** was reversed by the presence of constitutively expressed dominant-negative CREB mutant (A-CREB), suggesting that this phenomenon is a direct consequence of CREB activity.

L5 ANSWER 4 OF 10 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1999:203768 BIOSIS
DOCUMENT NUMBER: PREV199900203768
TITLE: Glucocorticoids stimulate CREB binding to a cyclic-AMP response element in the rat serine dehydratase gene.
AUTHOR(S): Haas, Michael J.; Pitot, Henry C. (1)
CORPORATE SOURCE: (1) McArdle Lab. Cancer Res., Dep. Oncol., Med. Sch., Univ. Wisconsin, 1400 University Ave., Madison, WI 53706 USA
SOURCE: Archives of Biochemistry and Biophysics, (Feb. 15, 1999) Vol. 362, No. 2, pp. 317-324.
ISSN: 0003-9861.
DOCUMENT TYPE: Article
LANGUAGE: English
AB Transcription of the rat serine dehydratase (SDH) gene, which is stimulated in hepatocytes by glucagon through the activity of the second messenger, cAMP, is augmented by pretreatment with glucocorticoids. A putative cAMP response element (CRE) located approximately 3.5 kbp upstream of the transcriptional start site was hypothesized to be responsible for this effect. Here we have demonstrated by DNaseI

footprinting and site-directed mutagenesis that the phosphorylated cAMP response element binding protein (CREB) binds to a cAMP response element different from that described previously. While the amount of CREB in the extracts is unaltered by hormone treatment, more CREB is capable of binding the response element upon addition of dexamethasone (Dex). These studies suggest that synergistic induction of the SDH gene by cAMP and Dex is through a CRE and is due, in part, to regulation of CREB-DNA binding by treatment of the cells with glucocorticoids.

L5 ANSWER 5 OF 10 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 1999255508 MEDLINE
DOCUMENT NUMBER: 99255508 PubMed ID: 10320759
TITLE: Forskolin-induced expression of tyrosine hydroxylase in human foetal brain cortex.
AUTHOR: Pliego Rivero F B; McCormack W J; Jauniaux E; Stern G M; Bradford H F
CORPORATE SOURCE: Imperial College of Science, Technology and Medicine, Department of Biochemistry, South Kensington, London SW7 2AY, UK.
SOURCE: BRAIN RESEARCH. DEVELOPMENTAL BRAIN RESEARCH, (1999 May 14) 114 (2) 201-6.
Journal code: DBR; 8908639. ISSN: 0165-3806.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199907
ENTRY DATE: Entered STN: 19990727
Last Updated on STN: 19990727
Entered Medline: 19990712

AB Brain-derived neurotrophic factor (BDNF) has previously been shown by this and other laboratories to work in concert with dopamine (DA) to induce the dopaminergic phenotype in foetal rat and human cerebral cortex during specified sensitive developmental stages. In the present study this induction by BDNF/DA was found to be greatly amplified by adding forskolin (fsk: 10 microM) to the rat and human cerebral cortex cultures together with DA (10 microM) and BDNF (50 ng/ml). This amplification was 14-fold for human tissue and 2-fold for rat tissue treated over an 80% shorter period. Compared to treatment with BDNF alone, the additional fsk increased tyrosine hydroxylase-positive (TH+) cell numbers by 220-fold in the human and 26-fold in the rat tissue. Parallel reverse transcription-polymerase chain reaction (RT-PCR) measurement of TH mRNA showed substantial increases above control levels when BDNF/DA or BDNF/DA/fsk treatments were applied. Since fsk boosts intracellular levels of cyclic AMP (cAMP), its amplifying action when added together with BDNF/DA is likely to be due to interactions via the cAMP response element/cAMP response element binding protein (**CRE/CREB**) systems. This is discussed.

L5 ANSWER 6 OF 10 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 1999216966 MEDLINE
DOCUMENT NUMBER: 99216966 PubMed ID: 10200909
TITLE: CREB-mediated transcriptional control.
AUTHOR: Andrisani O M
CORPORATE SOURCE: Department of Basic Medical Sciences, Purdue University, West Lafayette, IN 47907-1246, USA.
CONTRACT NUMBER: DK44533 (NIDDK)
SOURCE: CRITICAL REVIEWS IN EUKARYOTIC GENE EXPRESSION, (1999) 9 (1) 19-32. Ref: 132
Journal code: BEJ; 9007261. ISSN: 1045-4403.
PUB. COUNTRY: United States

Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199905
ENTRY DATE: Entered STN: 19990607
Last Updated on STN: 19990607
Entered Medline: 19990526

AB cAMP-response-element-binding protein, CREB, is a 43-kDa leucine zipper transcription factor identified and cloned via the study of cAMP-regulated genes. In the last decade, numerous studies have contributed much to our understanding of CREB structure, function, and CREB-mediated transcription. CREB binds to the cAMP-response-element (CRE) as a homodimer formed via the leucine zipper motif present at its C-terminus; its transcriptional activity is regulated by phosphorylation at Ser133, located within the N-terminal transactivation domain. Active, Ser133-phosphorylated CREB effects transcription of CRE-dependent genes via interaction with the 265-kDa co-activator protein CREB-binding-protein, CBP, which bridges the **CRE**/**CREB** complex to components of the basal transcriptional apparatus. This mechanism of CREB activation is effected by diverse signals, including those regulating the intracellular levels of cAMP and Ca+2, growth factors, and cellular stress. Accordingly, CREB-mediated transcription regulates diverse cellular responses, including intermediary metabolism, neuronal signaling, cell proliferation, and apoptosis. In addition to the regulation of CREB by phosphorylation, the viral oncoproteins HBV pX and HTLVI Tax regulate CREB transcriptional efficacy by an alternative mechanism, by increasing its DNA-binding affinity for viral and/or cellular CRE sites. In this review I describe key experiments that have defined the mechanism of CREB activation, with primary emphasis on emerging evidence linking CREB to cellular growth and development.

L5 ANSWER 7 OF 10 MEDLINE DUPLICATE 4
ACCESSION NUMBER: 96304560 MEDLINE
DOCUMENT NUMBER: 96304560 PubMed ID: 8722705
TITLE: Expression of the somatostatin gene and receptors in the rat harderian gland.
AUTHOR: Mato M E; Puig-Domingo M; Fornas O; Webb S M
CORPORATE SOURCE: Department of Endocrinology, Hospital de la Santa Creu i Sant Pau, Autonomous University of Barcelona, Spain.
SOURCE: MICROSCOPY RESEARCH AND TECHNIQUE, (1996 Jun 1) 34 (2) 118-22.
Journal code: BAG; 9203012. ISSN: 1059-910X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199611
ENTRY DATE: Entered STN: 19961219
Last Updated on STN: 19961219
Entered Medline: 19961108

AB Somatostatin is one of the numerous peptides described in the Harderian gland of different animals. With the aim of trying to elucidate its physiological role, we investigated whether this peptide is expressed in OFA rat Harderian gland at different ages and seasons and, if so, studied the regulatory proteins involved in the activation of the somatostatin gene, and also whether it contains any somatostatin receptors. Nursing (4-15-day-old), prepubertal (21-30-day-old), and adult (54-day-old) OFA rats were sacrificed by decapitation throughout the year, and the Harderian glands were excised and immediately frozen in liquid N2. The expression of somatostatin and its receptors was investigated using RT-PCR techniques; additionally, the existence of proteins which bind to cAMP responsive elements (CRE) was investigated using a band-shift technique. The somatostatin gene was expressed in the Harderian gland of rats aged 4-30 days in autumn and winter but not in spring and summer or in older

animals. Howev[redacted] the somatostatin receptor was [redacted]pressed throughout the year at all th[redacted]ges studied. In the autumn, nu[redacted]ear proteins binding to **CRE** (**CREB**) were present in 8-10-day-old rats but not in younger 4-day-old animals. We conclude that rat Harderian gland cells transcribe the somatostatin gene depending on the season and age of the animals, while its receptor is always present at all the ages studied; the CREB found produces the same retardation complex as ICER (inducible cAMP early repressor), an isoform of CREM (cAMP responsive element modulator), which in the pineal has been shown to be under adrenergic control. Since somatostatin expression is regulated by cAMP mechanisms, it is feasible that the existence of this repressor ICER could explain why somatostatin expression disappears in adult animals once maturation is complete.

L5 ANSWER 8 OF 10 MEDLINE DUPLICATE 5
ACCESSION NUMBER: 94253131 MEDLINE
DOCUMENT NUMBER: 94253131 PubMed ID: 8195196
TITLE: Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation.
AUTHOR: Enslen H; Sun P; Brickey D; Soderling S H; Klamo E; Soderling T R
CORPORATE SOURCE: Vollum Institute, Oregon Health Sciences University, Portland 97201.
CONTRACT NUMBER: DK44239 (NIDDK)
GM41292 (NIGMS)
SOURCE: JOURNAL OF BIOLOGICAL CHEMISTRY, (1994 Jun 3) 269 (22) 15520-7.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199406
ENTRY DATE: Entered STN: 19940707
Last Updated on STN: 19980206
Entered Medline: 19940630

AB We have characterized Ca2+/calmodulin-dependent protein kinase IV (CaM kinase IV), expressed using the baculovirus/Sf9 cell system, to assess its potential role in Ca2+-dependent transcriptional regulation. CaM kinase IV was strongly inhibited in vitro by KN-62, a specific CaM kinase inhibitor which suppresses Ca2+-dependent transcription of several genes, so we tested whether CaM kinase IV could stimulate transcription. Co-transfection of COS-1 cells by cDNA for CaM kinase IV gave 3-fold stimulation of a reporter gene expression, whereas co-transfection with CaM kinase II gave no transcriptional stimulation. Since this transcriptional response was mediated by phosphorylation of cAMP responsive element-binding protein (CREB), we determined the kinetics and site specificities of CaM kinases IV and II for phosphorylating CREB in vitro. CaM kinases IV and II and cAMP kinase (protein kinase A) all had similar Km values for CREB (1-5 microns), but the Vmax of CaM kinase IV was 40-fold lower than those of CaM kinase II and protein kinase A. Although all three kinases phosphorylated Ser133 in CREB, CaM kinase II also gave equal phosphorylation of a second site which was not Ser98. The two CREB phosphorylation sites were separately 32P-labeled, and the abilities of protein phosphatases 1, 2A, and 2B (calcineurin) to dephosphorylate them were tested. Our results show that all three phosphatases could dephosphorylate both sites, and calcineurin was a stronger catalyst for dephosphorylating site 1 (Ser133) than for site 2. These results indicate that CaM kinase IV may be important in Ca2+-dependent transcriptional regulation through phosphorylation of Ser133 in CREB. The fact that CaM kinase II phosphorylates another site in addition to Ser133 in CREB raises the possibility that this second phosphorylation site may account for the suppressed phosphorylation site may account for the suppressed ability of CaM kinase II to enhance transcription through the **CRE/CREB** system. In addition multiple protein phosphatases, including calcineurin, may exert a

modulatory eff[redacted] on transcription depending on [redacted]ich site they dephosphorylat[redacted]

L5 ANSWER 9 OF 10 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1994:473724 BIOSIS
DOCUMENT NUMBER: PREV199497486724
TITLE: Phosphorylation-regulated assembly of the cAMP-responsive enhancer (**CRE**):**CREB**:CBP complex.
AUTHOR(S): Lundblad, J. R. (1); Richards, J. P. (1); Loriaux, M. R. (1); Brennan, R. G.; Bachinger, H. P.; Goodman, R. H. (1)
CORPORATE SOURCE: (1) Vollum Inst., Oreg. Health Sci. Univ., Portland, OR 97201 USA
SOURCE: Society for Neuroscience Abstracts, (1994) Vol. 20, No. 1-2, pp. 634.
Meeting Info.: 24th Annual Meeting of the Society for Neuroscience Miami Beach, Florida, USA November 13-18, 1994
ISSN: 0190-5295.
DOCUMENT TYPE: Conference
LANGUAGE: English

L5 ANSWER 10 OF 10 MEDLINE DUPLICATE 6
ACCESSION NUMBER: 94301408 MEDLINE
DOCUMENT NUMBER: 94301408 PubMed ID: 7913207
TITLE: Nuclear protein CBP is a coactivator for the transcription factor CREB.
COMMENT: Comment in: Nature. 1994 Jul 21;370(6486):177
AUTHOR: Kwok R P; Lundblad J R; Chrivia J C; Richards J P; Bachinger H P; Brennan R G; Roberts S G; Green M R; Goodman R H
CORPORATE SOURCE: Vollum Institute, Oregon Health Sciences University, Portland 97201.
SOURCE: NATURE, (1994 Jul 21) 370 (6486) 223-6.
Journal code: NSC; 0410462. ISSN: 0028-0836.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199408
ENTRY DATE: Entered STN: 19940818
Last Updated on STN: 19970203
Entered Medline: 19940805

AB The transcription factor CREB binds to a DNA element known as the cAMP-regulated enhancer (**CRE**). **CREB** is activated through phosphorylation by protein kinase A (PKA), but precisely how phosphorylation stimulates CREB function is unknown. One model is that phosphorylation may allow the recruitment of coactivators which then interact with basal transcription factors. We have previously identified a nuclear protein of M(r)265K, CBP, that binds specifically to the PKA-phosphorylated form of CREB. We have used fluorescence anisotropy measurements to define the equilibrium binding parameters of the phosphoCREB:CBP interaction and report here that CBP can activate transcription through a region in its carboxy terminus. The activation domain of CBP interacts with the basal transcription factor TFIIB through a domain that is conserved in the yeast coactivator ADA-1 (ref. 8). Consistent with its role as a coactivator, CBP augments the activity of phosphorylated CREB to activate transcription of cAMP-responsive genes.

=> d his

(FILE 'HOME' ENTERED AT 15:38:38 ON 24 MAY 2001)

FILE 'MEDLINE, BIOSIS, BIOTECHNO' ENTERED AT 15:46:23 ON 24 MAY 2001
L1 588 S SUBUNIT OF PKA
L2 26 S L1 AND CRE

L3 11 DUP REM L2 (15 DUPLICATES REMOVED)
L4 19 S CREB
L5 10 DUP REM L4 (9 DUPLICATES REMOVED)

=> s l1 and cotransfect?

L6 47 L1 AND COTRANSFECT?

=> s l6 not l2

L7 41 L6 NOT L2

=> dup rem l7

PROCESSING COMPLETED FOR L7
L8 17 DUP REM L7 (24 DUPLICATES REMOVED)

=> s l8 and (amplif? or feedback)

L9 1 L8 AND (AMPLIF? OR FEEDBACK)

=> d ibib abs

L9 ANSWER 1 OF 1 MEDLINE
ACCESSION NUMBER: 97094694 MEDLINE
DOCUMENT NUMBER: 97094694 PubMed ID: 8939928
TITLE: Membrane localization of cAMP-dependent protein kinase **amplifies** cAMP signaling to the nucleus in PC12 cells.
AUTHOR: Cassano S; Gallo A; Buccigrossi V; Porcellini A; Cerillo R;
Gottesman M E; Avvedimento E V
CORPORATE SOURCE: Centro di Endocrinologia ed Oncologia Sperimentale del CNR,
c/o Dipartimento di Biologia e Patologia Molecolare e Cellulare, Facolta di Medicina, Universita "Federico II" 80131 Napoli, Italy.. Avvedim@cds.unina.it
SOURCE: JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 Nov 22) 271 (47) 29870-5.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199701
ENTRY DATE: Entered STN: 19970128
Last Updated on STN: 20000303
Entered Medline: 19970113

AB The A126 cell line, in contrast to its PC12 parent, does not differentiate, accumulate nuclear cAMP-dependent protein kinase A (PKA) catalytic subunit, or transcribe cAMP-dependent promoters in response to cAMP. Total PKA is reduced by 50% and is partly resistant to cAMP-induced dissociation in vivo. Unlike PC12, where PKAII is membrane-associated, PKAII is exclusively cytosolic in A126. **Cotransfection** with the RII anchor protein (AKAP75) and the PKA catalytic **subunit** (C-**PKA**) restored cAMP-induced transcription to levels found in PC12. These data indicate that membrane-bound PKAII **amplifies** cAMP signaling to the nucleus and suggest that cAMP-mediated responses are specified by the type and cellular localization of the PKA isoform.

=> d his

(FILE 'HOME' ENTERED AT 15:38:38 ON 24 MAY 2001)

FILE 'MEDLINE, BIOSIS, BIOTECHNO' ENTERED AT 15:46:23 ON 24 MAY 2001
L1 588 S SUBUNIT OF PKA

```
L2            26 S L1 AND CRE
L3            11 DUP REM L2 (15 DUPLICATES REMOVED)
L4            19 S CRE-CREB
L5            10 DUP REM L4 (9 DUPLICATES REMOVED)
L6            47 S L1 AND COTRANSFECT?
L7            41 S L6 NOT L2
L8            17 DUP REM L7 (24 DUPLICATES REMOVED)
L9             1 S L8 AND (AMPLIF? OR FEEDBACK)

=> d ibib abs l8 1-17


L8   ANSWER 1 OF 17  MEDLINE                                  DUPLICATE 1
ACCESSION NUMBER:    2000429946     MEDLINE
DOCUMENT NUMBER:     20392174   PubMed ID: 10933728
TITLE:               Regulation of the Epstein-Barr virus C promoter by AUF1
and
                     the cyclic AMP/protein kinase A signaling pathway.
AUTHOR:              Fuentes-Panana E M; Peng R; Brewer G; Tan J; Ling P D
CORPORATE SOURCE:    Department of Molecular Virology and Microbiology, Baylor
                     College of Medicine, Houston, Texas 77030, USA.
CONTRACT NUMBER:     R29CA69437 (NCI)
                     RO1CA52443 (NCI)
SOURCE:              JOURNAL OF VIROLOGY, (2000 Sep) 74 (17) 8166-75.
                     Journal code: KCV; 0113724. ISSN: 0022-538X.
PUB. COUNTRY:        United States
                     Journal; Article; (JOURNAL ARTICLE)
LANGUAGE:            English
FILE SEGMENT:        Priority Journals
ENTRY MONTH:         200009
ENTRY DATE:          Entered STN: 20000922
                     Last Updated on STN: 20000922
                     Entered Medline: 20000914
AB   EBNA2 is an Epstein-Barr virus (EBV)-encoded protein that regulates the
     expression of viral and cellular genes required for EBV-driven B-cell
     immortalization. Elucidating the mechanisms by which EBNA2 regulates
viral
     and cellular gene expression is necessary to understand EBV-induced
B-cell
     immortalization and viral latency in humans. EBNA2 targets to the latency
     C promoter (Cp) through an interaction with the cellular DNA binding
     protein CBF1 (RBPJk). The EBNA2 enhancer in Cp also binds another
cellular
     factor, C promoter binding factor 2 (CBF2), whose protein product(s) has
     not yet been identified. Within the EBNA2 enhancer in Cp, we have
     previously identified the DNA sequence required for CBF2 binding and also
     determined that this element is required for efficient activation of Cp
by
     EBNA2. In this study, the CBF2 activity was biochemically purified and
     microsequenced. The peptides sequenced were identical to the hnRNP
protein
     AUF1. Antibodies against AUF1 but not antibodies to related hnRNP
proteins
     reacted with CBF2 in gel mobility shift assays. In addition, stimulation
     of the cellular cyclic AMP (cAMP)/protein kinase A (PKA) signal
     transduction pathway results in an increase in detectable CBF2/AUF1
     binding activity extracted from stimulated cells. Furthermore, the CBF2
     binding site was able to confer EBNA2 responsiveness to a heterologous
     promoter when transfected cells were treated with compounds that activate
     PKA or by cotransfection of plasmids expressing a constitutively
     active catalytic subunit of PKA. EBNA2-mediated
     stimulation of the latency Cp is also increased in similar
     cotransfection assays. These results further support an important
     role for CBF2 in mediating EBNA2 transactivation; they identify the hnRNP
     protein AUF1 as a major component of CBF2 and are also the first evidence
     of a cis-acting sequence other than a CBF1 binding element that is able
to
     confer responsiveness to EBNA2.
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L8 ANSWER 2 OF 17 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 20095040 MEDLINE
DOCUMENT NUMBER: 20095040 PubMed ID: 10627585
TITLE: Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase.
AUTHOR: Banke T G; Bowie D; Lee H; Huganir R L; Schousboe A; Traynelis S F
CORPORATE SOURCE: Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322, USA.
SOURCE: JOURNAL OF NEUROSCIENCE, (2000 Jan 1) 20 (1) 89-102.
Journal code: JDF; 8102140. ISSN: 1529-2401.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200001
ENTRY DATE: Entered STN: 20000204
Last Updated on STN: 20010521
Entered Medline: 20000127

AB Modulation of postsynaptic AMPA receptors in the brain by phosphorylation may play a role in the expression of synaptic plasticity at central excitatory synapses. It is known from biochemical studies that GluR1 AMPA receptor subunits can be phosphorylated within their C terminal by cAMP-dependent protein kinase A (PKA), which is colocalized with the phosphatase calcineurin (i.e., phosphatase 2B). We have examined the effect of PKA and calcineurin on the time course, peak open probability (P(O, PEAK)), and single-channel properties of glutamateevoked responses for neuronal AMPA receptors and homomeric GluR1(flip) receptors recorded in outside-out patches. Inclusion of purified catalytic **subunit** Calpha-**PKA** in the pipette solution increased neuronal AMPA receptor P(O,PEAK) (0.92) compared with recordings made with calcineurin included in the pipette (P(O,PEAK) 0.39). Similarly, Calpha-PKA increased P(O,PEAK) for recombinant GluR1 receptors (0. 78) compared with patches excised from cells **cotransfected** with a cDNA encoding the PKA peptide inhibitor PKI (P(O,PEAK) 0.50) or patches with calcineurin included in the pipette (P(O,PEAK) 0.42). Neither PKA nor calcineurin altered the amplitude of single-channel subconductance levels, weighted mean unitary current, mean channel open period, burst length, or macroscopic response waveform for recombinant GluR1 receptors. Substitution of an amino acid at the PKA phosphorylation site (S845A) on GluR1 eliminated the PKA-induced increase in P(O,PEAK), whereas the mutation of a Ca(2+), calmodulin-dependent kinase II and PKC phosphorylation site (S831A) was without effect. These results suggest that AMPA receptor peak response open probability can be increased by PKA through phosphorylation of GluR1 Ser845.

L8 ANSWER 3 OF 17 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 2000068716 MEDLINE
DOCUMENT NUMBER: 20068716 PubMed ID: 10600163
TITLE: Transcriptional regulation of 5-aminolevulinate synthase by
phenobarbital and cAMP-dependent protein kinase.
AUTHOR: Varone C L; Giono L E; Ochoa A; Zakin M M; Canepa E T
CORPORATE SOURCE: Departamento de Quimica Biologica, Universidad de Buenos Aires, Buenos Aires, Argentina.
SOURCE: ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, (1999 Dec 15) 372 (2) 261-70.
Journal code: 6SK; 0372430. ISSN: 0003-9861.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200001
ENTRY DATE: Entered STN: 20000204
Last Updated on STN: 20000204
Entered Medline: 20000124

AB 5-Aminolevulinate synthase (ALA-S) is a mitochondrial matrix enzyme that catalyzes the first and rate-limiting step of the heme biosynthesis. There

are two ALA-S isozymes encoded by distinct genes. One gene encodes an isozyme that is expressed exclusively in erythroid cells, and the other gene encodes a housekeeping isozyme that is apparently expressed in all tissues. In this report we examine the mechanisms by which phenobarbital and cAMP regulate housekeeping ALA-S expression. We have determined that cAMP and phenobarbital effects are additive and the combined action is necessary to observe the cAMP effect on ALA-S mRNA in rat hepatocytes. The role of the cAMP-dependent protein kinase (PKA) has been examined. A synergism effect on ALA-S mRNA induction is observed in rat hepatocytes treated with pairs of selective analogs by each PKA cAMP binding sites. A 870-bp fragment of ALA-S 5'-flanking region is able to provide cAMP and phenobarbital stimulation to chloramphenicol O-acetyltranferase fusion vectors in transiently transfected HepG2 cells. ALA-S promoter activity is induced by **cotransfection** with an expression vector containing the catalytic **subunit** of **PKA**. Furthermore, **cotransfection** with a dominant negative mutant of the PKA regulatory subunit impairs the cAMP analog-mediated increase, but the phenobarbital-mediated induction is not modified. Our data suggest that the transcription factor cAMP-response element binding protein (CREB) is probably involved in PKA induction of ALA-S gene expression. Finally, heme addition greatly decreases the basal and phenobarbital or cAMP analog-mediated induction of ALA-S promoter activity. The present work provides evidence that cAMP, through PKA-mediated CREB phosphorylation, and phenobarbital induce ALA-S expression at the transcriptional level, while heme represses it.

L8 ANSWER 4 OF 17 MEDLINE DUPLICATE 4
ACCESSION NUMBER: 1999316039 MEDLINE
DOCUMENT NUMBER: 99316039 PubMed ID: 10385697
TITLE: Both inducible and constitutive activator protein-1-like transcription factors are used for transcriptional activation of the galanin gene by different first and second messenger pathways.
AUTHOR: Anouar Y; Lee H W; Eiden L E
CORPORATE SOURCE: Section on Molecular Neuroscience, Laboratory of Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, Maryland, USA.
SOURCE: MOLECULAR PHARMACOLOGY, (1999 Jul) 56 (1) 162-9.
Journal code: NGR; 0035623. ISSN: 0026-895X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199907
ENTRY DATE: Entered STN: 19990730
Last Updated on STN: 19990730
Entered Medline: 19990722

AB We investigated trans-acting factors mediating galanin (GAL) gene activation by protein kinase-dependent signal transduction pathways in chromaffin cells. GAL mRNA up-regulation via the protein kinase A (PKA) pathway (25 microM forskolin) required new protein synthesis. Stimulation via protein kinase C (0.1 microM phorbol myristate acetate) did not. The involvement of activator protein-1(AP-1) and cAMP response element-binding protein (CREB) in serine/threonine protein kinase activation of GAL gene transcription was assessed. **Cotransfection** of a GAL reporter gene along with expression plasmids encoding c-Jun plus c-Fos, or the catalytic **subunit** of **PKA** (PKAbeta), resulted in a 4- to 8-fold enhancement of GAL reporter gene transcription. Transcriptional activation required the galanin 12-O-tetradecanoylphorbol-13-acetate (phorbol-12-myristate-13-acetate) response element (GTRE) octamer sequence (TGACGCGG) in the proximal enhancer of the GAL gene, previously shown to confer phorbol ester responsiveness in chromaffin cells. CREB coexpression

did not stimula[redacted] GAL gene transcription or inc[redacted]e transcriptional activation by [redacted]beta. The GTRE preferentially bound in vitro synthesized Jun and Fos-Jun, compared with CREB, in electrophoretic mobility shift assays. The GTRE preference for binding AP-1-immunoreactive protein compared with CREB was even more pronounced in chromaffin cell nuclear extracts, in which the majority of GTRE-bound protein in electrophoretic mobility shift assays was supershifted with anti-Fos and anti-Jun antibodies. Thus, GAL gene regulation mediated by protein kinase activation appears to involve both constitutively expressed and inducible AP-1-related proteins. Elevated potassium stimulation of GAL mRNA was completely blocked, but pituitary adenylyl cyclase-activating polypeptide and histamine stimulations were only partially blocked, by cycloheximide. Both inducible and constitutive pathways are therefore used by physiologically relevant first messengers that stimulate GAL biosynthesis in vivo.

L8 ANSWER 5 OF 17 BIOTECHNO COPYRIGHT 2001 Elsevier Science B.V.
ACCESSION NUMBER: 1998:28319681 BIOTECHNO
TITLE: Evidence that protein kinase A activity is required for the basal and tax-stimulated transcriptional activity of human T-cell leukemia virus type- I long terminal repeat
AUTHOR: Turgeman H.; Aboud M.
CORPORATE SOURCE: M. Aboud, Dept. of Microbiology and Immunology, Faculty of Health Sciences, Ben Gurion University of Negev, Beer Sheva 84105, Israel.
E-mail: aboud@bgumail.bgu.ac.il
SOURCE: FEBS Letters, (29 MAY 1998), 428/3 (183-187), 40 reference(s)
CODEN: FEBLAL ISSN: 0014-5793
PUBLISHER ITEM IDENT.: S0014579398005134
DOCUMENT TYPE: Journal; Article
COUNTRY: Netherlands
LANGUAGE: English
SUMMARY LANGUAGE: English
AN 1998:28319681 BIOTECHNO
AB The present study was undertaken to investigate the role of protein kinase A (PKA) in the control of human T-cell leukemia virus type-I (HTLV-I) long terminal repeat (LTR) expression, since this issue is still controversial. For this purpose we employed two human T-cell lines; the Jurkat cells in which long exposure to diBu-cAMP severely down-regulated the catalytic **subunit of PKA** (PKA-C), and H-9 cells in which such exposure markedly increased PKA-C level. Transient transfection assays revealed that addition of diBu-cAMP 1 h before or after transfection profoundly increased HTLV-I LTR directed CAT expression and synergistically enhanced its stimulation by the viral transactivator tax gene product in both cell lines. However longer exposure to diBu-cAMP before transfection reduced LTR-CAT expression to below its basal level and completely abolished its stimulation by tax in Jurkat cells, and this diBu-cAMP inhibitory effect could be abrogated by **cotransfection** of a PKA-C expressing vector. By contrast, in H- 9 cells, this long exposure to diBu-cAMP continued enhancing LTR-CAT expression and its tax-mediated transactivation, and this stimulatory effect of diBu-cAMP could be diminished by the PKA-specific inhibitor N-12-(p- bromocinnamylamine)ethyl!-5-isoquinolinsulfonamide (H-89). Notably, in the absence of diBu-cAMP treatment H-89 reduced LTR-CAT expression to below its basal level and prevented its stimulation by tax in both cell lines. Together these findings indicate not only that cAMP-activated PKA stimulates HTLV-I LTR expression and its transactivation by tax, but even in the absence of PKA activating signals the basal HTLV-I LTR expression as well as its stimulation by tax are both dependent on a basal PKA activity.

L8 ANSWER 6 OF 17 MEDLINE DUPLICATE 5
ACCESSION NUMBER: 97433268 MEDLINE
DOCUMENT NUMBER: 97433268 PubMed ID: 9288908

TITLE: [redacted]termination of functional doma[redacted] of the human transcription factor PAX8 responsible for its nuclear localization and transactivating potential.
AUTHOR: Poleev A; Okladnova O; Musti A M; Schneider S; Royer-Pokora B; Plachov D
CORPORATE SOURCE: Institute for Cell Biology, University Clinic, Essen, Germany.
SOURCE: EUROPEAN JOURNAL OF BIOCHEMISTRY, (1997 Aug 1) 247 (3) 860-9.
Journal code: EMZ; 0107600. ISSN: 0014-2956.
PUB. COUNTRY: GERMANY: Germany, Federal Republic of
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199710
ENTRY DATE: Entered STN: 19971013
Last Updated on STN: 20000303
Entered Medline: 19971002

AB The conserved structure of the transcription factors of the Pax gene family may reflect functional conservation. We have demonstrated that the human Pax8 transcription factor is organized in several functional domains and contains two regions responsible for its nuclear localization, in addition to an activating region at the carboxy terminus of the protein and an inhibitory region encoded by the exon 9 present only in a splice variant PAX8a. Regions of PAX8 determining the nuclear localization of the PAX8A/lacZ fusions contain short amino acid sequences similar to several described nuclear localization sites (NLS). These NLS were identified in the paired domain and between the octapeptide and the residual homeodomain, respectively. The activating domain is encoded by the exons 10 and 11 and its function is modulated by the adjacent domains encoded by the exons 9 and 12. The domain encoded by exon 9 significantly inhibits the function of the activating domain. Pax8 is expressed in thyroid cells and its product binds promoters of the thyroglobulin and thyroperoxidase genes through its paired domain. Thyroid cell growth and differentiation depend on thyrotropin which, by stimulating cAMP synthesis, activates the cAMP-dependent protein kinase A (PKA). We have investigated a link between thyrotropin stimulation and gene activation by Pax8. Stimulation of cAMP synthesis augments Pax8-specific transcription in thyroid cells, indicating that PKA is involved in Pax8 activation. **Cotransfection** of GAL4/PAX8 fusions and the catalytic **subunit** of **PKA** in A126, a PKA-deficient derivative of the PC12 pheochromocytoma cell line, synergistically activates the GAL4-specific reporter, suggesting the activating domain of PAX8 is dependent upon the catalytic subunit of the PKA. We propose that this dependence is due to a hypothetical adaptor which forms a target for PKA and interacts with the activating domain of PAX8. We show that PAX8 isolated from the thyroid cell line FTRL5 is a phosphoprotein in which phosphorylation is not dependant on cAMP pathway activation. Our results suggest that Pax8 is part of the cAMP signaling pathway and mediates thyrotropin-dependent gene activation in thyroid cells. Investigation of the PAX8 expression in a panel of Wilms' tumors shows a striking correlation between the expression of PAX8 and another transcription factor, WT1, indicating that these two genes may interact in vivo.

L8 ANSWER 7 OF 17 MEDLINE DUPLICATE 6
ACCESSION NUMBER: 97094694 MEDLINE
DOCUMENT NUMBER: 97094694 PubMed ID: 8939928
TITLE: Membrane localization of cAMP-dependent protein kinase amplifies cAMP signaling to the nucleus in PC12 cells.
AUTHOR: Cassano S; Gallo A; Buccigrossi V; Porcellini A; Cerillo R; Gottesman M E; Avvedimento E V

CORPORATE SOURCE: [redacted]ntro di Endocrinologia ed Onco[redacted]ia Sperimentale del CNR,
c/o Dipartimento di Biologia e Patologia Molecolare e Cellulare, Facolta di Medicina, Universita "Federico II" 80131 Napoli, Italy.. Avvedim@cds.unina.it
SOURCE: JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 Nov 22) 271 (47) 29870-5.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199701
ENTRY DATE: Entered STN: 19970128
Last Updated on STN: 20000303
Entered Medline: 19970113

AB The A126 cell line, in contrast to its PC12 parent, does not differentiate, accumulate nuclear cAMP-dependent protein kinase A (PKA) catalytic subunit, or transcribe cAMP-dependent promoters in response to cAMP. Total PKA is reduced by 50% and is partly resistant to cAMP-induced dissociation in vivo. Unlike PC12, where PKAII is membrane-associated, PKAII is exclusively cytosolic in A126. **Cotransfection** with the RII anchor protein (AKAP75) and the PKA catalytic **subunit** (C-**PKA**) restored cAMP-induced transcription to levels found in PC12. These data indicate that membrane-bound PKAII amplifies cAMP signaling to the nucleus and suggest that cAMP-mediated responses are specified by the type and cellular localization of the PKA isoform.

L8 ANSWER 8 OF 17 MEDLINE DUPLICATE 7
ACCESSION NUMBER: 96326623 MEDLINE
DOCUMENT NUMBER: 96326623 PubMed ID: 8706900
TITLE: Down-regulation of the protein kinase A pathway by activators of protein kinase C and intracellular Ca2+ in fibroblast cells.
AUTHOR: Dobbeling U; Berchtold M W
CORPORATE SOURCE: Institut fur Veterinarbiochemie, Universitat Zurich, Switzerland.
SOURCE: FEBS LETTERS, (1996 Aug 5) 391 (1-2) 131-3.
Journal code: EUH; 0155157. ISSN: 0014-5793.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199609
ENTRY DATE: Entered STN: 19960919
Last Updated on STN: 19980206
Entered Medline: 19960912

AB Many genes are regulated by the intracellular calcium, protein kinase C (PKC) and protein kinase A (PKA) pathways and it has been shown that these pathways synergize in some cell types, whereas they antagonize in others. Here we show that the calcium and PKC pathways suppress the effects mediated by the PKA pathway in a fibroblast cell line. The suppressing effect of elevated intracellular Ca2+ levels, but not of the PKC pathway, can be abrogated by the addition of cyclosporin A (CsA), indicating that the effect of Ca2+ is mediated by phosphatase-2B (PP-2B/calcineurin). Suppression by the PKC pathway is not mediated by the proto-oncogenes c-fos, c-jun and junB, as the co-transfection of these genes does not block the effects of the PKA stimulator 8-Br-cAMP. In addition, **cotransfection** with the catalytic **subunit** of **PKA** shows that the inhibitory effect of PKC occurs upstream of PKA activation.

L8 ANSWER 9 OF 17 MEDLINE DUPLICATE 8
ACCESSION NUMBER: 95377220 MEDLINE
DOCUMENT NUMBER: 95377220 PubMed ID: 7649098
TITLE: Regulation of insulin-like growth factor I transcription by
cyclic adenosine 3',5'-monophosphate (cAMP) in fetal rat

bone cells through an element within exon 1: protein kinase A-dependent control without a consensus AMP response element.

AUTHOR: McCarthy T L; Thomas M J; Centrella M; Rotwein P

CORPORATE SOURCE: Section of Plastic Surgery, Yale University School of Medicine, New Haven, Connecticut 06520-8041, USA.

CONTRACT NUMBER: DK-37449 (NIDDK)
DK-47421 (NIDDK)
HD-20805 (NICHD)

SOURCE: ENDOCRINOLOGY, (1995 Sep) 136 (9) 3901-8.
Journal code: EGZ; 0375040. ISSN: 0013-7227.

PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English

FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals

ENTRY MONTH: 199509

ENTRY DATE: Entered STN: 19951005
Last Updated on STN: 19951005
Entered Medline: 19950922

AB Insulin-like growth factor I (IGF-I) is a locally synthesized anabolic growth factor for bone. IGF-I synthesis by primary fetal rat osteoblasts (Ob) is stimulated by agents that increase the intracellular cAMP concentration, including prostaglandin E2 (PGE2). Previous studies with Ob cultures demonstrated that PGE2 enhanced IGF-I transcription through selective use of IGF-I promoter 1, with little effect on IGF-I messenger RNA half-life. Transient transfection of Ob cultures with an array of promoter 1-luciferase reporter fusion constructs has now allowed localization of a potential cis-acting promoter element(s) responsible for cAMP-stimulated gene expression to the 5'-untranslated region (5'-UTR) of IGF-I exon 1, within a segment lacking a consensus cAMP response element. Our evidence derives from three principal observations: 1) a transfection construct containing only 122 nucleotides (nt) of promoter 1 and 328 nt of the 5'-UTR retained full PGE2-stimulated reporter expression; 2) maximal PGE2-driven reporter expression required the presence of nt 196 to 328 of exon 1 when tested within the context of IGF-I promoter 1; 3) **cotransfection** of IGF-I promoter-luciferase-reporter constructs with a plasmid encoding the alpha-isoform of the catalytic subunit of murine cAMP-dependent protein kinase (PKA) produced results comparable to those seen with PGE2 treatment, whereas **cotransfection** with a plasmid encoding a mutant regulatory **subunit** of **PKA** that cannot bind cAMP blocked PGE2-induced reporter expression. Deoxyribonuclease I footprinting of the 5'-UTR of exon 1 demonstrated protected sequences at HS3A, HS3B, and HS3D, three of six DNA-protein binding sites previously characterized with rat liver nuclear extracts. Of these three regions, only the HS3D binding site is located within the functionally identified hormonally responsive segment of IGF-I exon 1. These results directly implicate PKA in the control of IGF-I gene transcription by PGE2 and identify a segment of IGF-I exon 1 as being essential for this hormonal regulation.

L8 ANSWER 10 OF 17 MEDLINE DUPLICATE 9

ACCESSION NUMBER: 96082465 MEDLINE

DOCUMENT NUMBER: 96082465 PubMed ID: 7476971

TITLE: Pituitary-type transcription of the human prolactin gene in the absence of Pit-1.

AUTHOR: Gellersen B; Kempf R; Telgmann R; DiMattia G E

CORPORATE SOURCE: Institute for Hormone and Fertility Research, University of Hamburg, Germany.

SOURCE: MOLECULAR ENDOCRINOLOGY, (1995 Jul) 9 (7) 887-901.
Journal code: NGZ; 8801431. ISSN: 0888-8809.

PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199512
ENTRY DATE: Entered STN: 19960124
Last Updated on STN: 19970203
Entered Medline: 19951212

AB We describe a human (h) PRL-producing cell line, SKUT-1B-20, which we isolated as a subclone of a uterine sarcoma cell line. Although this cell line is of uterine origin, it does not use the decidual-specific upstream promoter of the hPRL gene, but transcribes the hPRL gene from the downstream pituitary-type transcription start site, as determined by Northern blot, reverse transcriptase-polymerase chain reaction and primer extension analyses. This is particularly intriguing because SKUT-1B-20 cells lack the transcription factor Pit-1. No Pit-1 messenger RNA was detectable by reverse transcriptase-polymerase chain reaction, and endogenous Pit-1 target genes (GH, PRL, and Pit-1) were refractory to transfected Pit-1 expression vector, whereas in **cotransfection** experiments, Pit-1 efficiently activated reporter gene fusion constructs carrying 5'-flanking sequences of the human and rat PRL or the mouse Pit-1
genes. By transfecting reporter genes containing 8.7 kilobases of DNA flanking the hPRL pituitary-specific start site (hPRL-8700/Luc) and deletions thereof, we located a Pit-1-independent cis-active region more than 7 kilobases upstream of the start site. The most distal 1650 or 880 base pairs of the hPRL genomic fragment (which extends to -8784 base pairs), when placed directly upstream of the homologous hPRL or the heterologous thymidine kinase promoters, conferred transcriptional activation to those promoters. SKUT-1B-20 cell-specific activation of hPRL-8700/Luc could not be suppressed by the introduction of an inhibitor of protein kinase A (PKA), PKI. This is the first demonstration of pituitary-type PRL gene transcription independent of Pit-1 and activation of the PKA pathway. The SKUT-1B-20 cell line was then used in reconstitution experiments to delineate the role of Pit-1 in modulating the transcriptional effects of phorbol ester, PKA, and estrogen receptor (ER) on the hPRL gene. The low response of hPRL/luciferase fusion genes to
phorbol ester was greatly enhanced by **cotransfected** Pit-1 and was mediated by the proximal region between -250 and -38. The catalytic **subunit** of **PKA**, C beta, was able to elicit a moderate induction of hPRL-8700/Luc even in the absence of Pit-1. A potential estrogen response element has been located in the hPRL gene sequence at a position similar to that of the estrogen response element of the rat PRL gene immediately adjacent to the distal enhancer. (ABSTRACT TRUNCATED AT 400 WORDS)

L8 ANSWER 11 OF 17 MEDLINE DUPLICATE 10
ACCESSION NUMBER: 95118911 MEDLINE
DOCUMENT NUMBER: 95118911 PubMed ID: 7819133
TITLE: Response of the phosphoenolpyruvate carboxykinase gene to glucocorticoids depends on the integrity of the cAMP pathway.
AUTHOR: Angrand P O; Coffinier C; Weiss M C
CORPORATE SOURCE: Unite de Genetique de la Differenciation, URA 1149 du Centre National de la Recherche Scientifique, Departement de Biologie Moleculaire, Institut Pasteur, Paris, France.
SOURCE: CELL GROWTH AND DIFFERENTIATION, (1994 Sep) 5 (9) 957-66.

Journal code: AYH; 9100024. ISSN: 1044-9523.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199502
ENTRY DATE: Entered STN: 19950223
Last Updated on STN: 19980206
Entered Medline: 19950214

AB The phosphoenolpyruvate carboxykinase (PEPCK) gene is regulated at the transcriptional level by a variety of effectors in a tissue-specific fashion. In order to study the parameters involved in the tissue-specific

hormonal regulation of the PEPCK gene, we have used a transient expression test in well-differentiated rat hepatoma cells as well as in dedifferentiated variants. In this test, the PEPCK promoter is induced by glucocorticoids in well-differentiated FGC4 cells, but not in H5 dedifferentiated variants, in spite of the presence in H5 cells of the glucocorticoid receptor. Study of the PEPCK promoter using electrophoretic mobility shift assays reveals binding sites for the liver-enriched transcription factors HNF1, vHNF1, HNF3, HNF4, and CAAT/enhancer binding protein members. Overexpression of the liver-enriched transcription factors absent in the dedifferentiated variants, such as HNF1 and HNF4, is not sufficient to restore glucocorticoid response of the PEPCK promoter in the variants. Moreover, systematic analysis of the PEPCK promoter reveals that the presence of a region covering a cAMP-responsive element (CRE1 at -80) and a CAAT box is necessary for full response of the PEPCK promoter to glucocorticoids in well-differentiated rat hepatoma cells. In a **cotransfection** test, overexpression of the regulatory subunit of protein kinase A (PKA), causing sequestering of PKA, abolishes the glucocorticoid response of the promoter in well-differentiated cells. On the other hand, in dedifferentiated variants, overexpression of the catalytic **subunit** of **PKA** restores the response to glucocorticoids. The action of PKA on the glucocorticoid response requires the presence of the CRE1 element and is promoter specific because it does not concern nonhepatic promoters such as the long terminal repeats of the mouse mammary tumor virus. These results suggest that the full response of the PEPCK promoter to glucocorticoids requires activation of another signal transduction pathway, the cAMP-mediated pathway.

L8 ANSWER 12 OF 17 MEDLINE DUPLICATE 11

ACCESSION NUMBER: 94076026 MEDLINE
DOCUMENT NUMBER: 94076026 PubMed ID: 8254371
TITLE: PKA-dependent regulation of mKv1.1, a mouse Shaker-like potassium channel gene, when stably expressed in CHO cells.
AUTHOR: Bosma M M; Allen M L; Martin T M; Tempel B L
CORPORATE SOURCE: Geriatric Research Education and Clinical Center, VA Medical Center, Seattle, Washington 98108.
CONTRACT NUMBER: HL-44948 (NHLBI)
NS-27206 (NINDS)
SOURCE: JOURNAL OF NEUROSCIENCE, (1993 Dec) 13 (12) 5242-50.
Journal code: JDF; 8102140. ISSN: 0270-6474.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199401
ENTRY DATE: Entered STN: 19940203
Last Updated on STN: 19940203
Entered Medline: 19940112

AB Potassium (K) channels are important regulators of cellular physiology and can themselves be modulated by phosphorylation. We have investigated the potential protein kinase A (PKA) regulation of mKv1.1, a mouse Shaker-like K channel gene, when it is expressed in stably transfected Chinese hamster ovary (CHO) cell lines. Whole-cell patch-clamp records show that expression of mKv1.1 gives rise to a rapidly activating, sustained K+ current, referred to classically as a delayed rectifier-type current. In order to study the effects of PKA, we compared cell lines transfected with mKv1.1 alone with lines **cotransfected** with both mKv1.1 and a plasmid encoding a dominant negative mutation in the regulatory **subunit** of **PKA**. These mutant regulatory subunits bind to

endogenous catalytic subunits of PKA but do not respond to cAMP, thereby causing a chronic reduction in the basal PKA activity in these cells. We found that mKv1.1 current kinetics are unaltered but current density is 3.4-fold higher in the cell lines expressing mutant regulatory subunit than in lines expressing only mKv1.1. RNase protection assays indicate that levels of the specific RNA for mKv1.1 are increased almost twofold in the lines expressing mutant regulatory subunit over the lines expressing mKv1.1 only. Further, the levels of mKv1.1 protein, assayed using an mKv1.1 channel-specific antibody, are increased by almost a factor of 3 between the two types of cell lines. These results suggest that PKA can regulate mKv1.1 channel expression by changing steady-state levels of RNA and by other posttranscriptional mechanisms.

L8 ANSWER 13 OF 17 MEDLINE DUPLICATE 12
ACCESSION NUMBER: 93234519 MEDLINE
DOCUMENT NUMBER: 93234519 PubMed ID: 7682705
TITLE: A dual role for the cAMP-dependent protein kinase in tyrosine hydroxylase gene expression.
AUTHOR: Kim K S; Park D H; Wessel T C; Song B; Wagner J A; Joh T H
CORPORATE SOURCE: Laboratory of Molecular Neurobiology, W. M. Burke Medical Research Institute, Cornell University Medical College, White Plains, NY 10605.
CONTRACT NUMBER: MH24285 (NIMH)
MH48866 (NIMH)
SOURCE: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1993 Apr 15) 90 (8) 3471-5. Journal code: PV3; 7505876. ISSN: 0027-8424.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199305
ENTRY DATE: Entered STN: 19930604
Last Updated on STN: 19980206
Entered Medline: 19930517

AB Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to 3,4-dihydroxy-L-phenylalanine, the first and rate-limiting step in catecholamine biosynthesis. The cAMP-dependent protein kinase (PKA) phosphorylates and activates the TH enzyme and is thought to mediate transcriptional induction of the TH gene. To better understand the functional role of PKA in TH gene regulation, we studied TH gene expression at the transcriptional, translational, and post-translational levels in several PKA-deficient cell lines derived from rat PC12 pheochromocytoma cells. Strikingly, all PKA-deficient cell lines analyzed in this study showed substantial deficits in basal TH expression as measured by TH enzymatic activity, level of TH immunoreactivity, TH protein level, and steady-state mRNA level. Interestingly, the steady-state level of mRNA correlated well with levels of TH activity, immunoreactivity, and protein. In addition, PKA-deficient cell lines lacked transcriptional induction of the TH gene following treatment with dibutyryl cAMP. **Cotransfection** of PKA-deficient cells with an expression plasmid for the catalytic **subunit** of **PKA** fully reversed transcriptional defect, as indicated by robust transcriptional induction of a reporter construct containing 2400 bp of TH upstream sequence in all PC12 cells tested. These data indicate that the PKA system regulates both the basal and the cAMP-inducible expression of the TH gene primarily at the transcriptional level in PC12 cells.

L8 ANSWER 14 OF 17 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1992:482909 BIOSIS
DOCUMENT NUMBER: BA94:114284
TITLE: STIMULATION OF PRODYNORPHIN GENE EXPRESSION REQUIRES A FUNCTIONAL PROTEIN KINASE A.
AUTHOR(S): KAYNARD A H; MELNER M H
CORPORATE SOURCE: DIVISION NEUROSCIENCE, OREGON REGIONAL PRIMATE RESEARCH CENTER, 505 N.W. 185TH AVENUE, BEAVERTON, OREG. 97006.
SOURCE: MOL CELL NEUROSCI, (1992) 3 (4), 278-285.

CODEN: MOCNED. ISSN: 1044-7431.
FILE SEGMENT: A; OLD
LANGUAGE: English
AB To investigate the involvement of the cAMP/protein kinase A (PKA) second messenger system in mediating the hormonal regulation of prodynorphin expression, a normal, homologous cell transfection model was used. Ovarian granulosa cells, which express high levels of prodynorphin mRNA in response to hormonal stimulation, were obtained from the ovaries of gonadotropin-primed 26-day-old rats, pooled, and cultured under serum-free conditions. Cells were transfected with a plasmid construct (proDYN-CAT) containing .apprx.2 kb of the rat prodynorphin gene (-1858 to +133 bp relative to the transcription start site) fused to the chloramphenicol acetyltransferase (CAT) reporter gene. Cultures were **cotransfected** with either a control plasmid (pUC13) or the Mt-REV expression plasmid; Mt-REV overexpresses a mutated form of the RI.alpha. regulatory **subunit** of **PKA** which binds to and thereby inhibits the activity of the catalytic subunit. When granulosa cells were **cotransfected** with proDYN-CAT and a control plasmid, CAT activity was stimulated by human FSH (20 ng/ml), human chorionic gonadotropin (10 ng/ml), and 8-(4-chlorphenyl thio)-cAMP (0.5 mM) (10-, 11-, and 6-fold, respectively; $P < 0.005$). However, when cells were **cotransfected** with proDYN-CAT and Mt-REV there was a complete abolition of hormone and cAMP stimulation of CAT activity ($P > 0.4$ vs controls). The effect of Mt-REV **cotransfection** was specific since contransfection with Mt-REV wt, a plasmid which ovrexpresses the normal, wild-type regulatory subunit, had no effect on hormonal responsiveness. Furthermore, **cotransfection** with Mt-C.alpha.EV or Mt-C.beta.EV (which overexpress the .alpha. or .beta. catalytic subunits of PKA and thus mimic the effects of PKA activation) significantly stimulated prodynorphin promoter activity by 14-fold ($P < 0.05$). These results suppport the hypothesis that gonadotropin stimulation of granulosa cells increases prodynorphin promoter activity via a process which requires a functional PKA second messenger system. Thus, PKA may be a generalized mechanism by which stimulatory input increases cellular prodynorphin expression.

L8 ANSWER 15 OF 17 MEDLINE DUPLICATE 13
ACCESSION NUMBER: 91268059 MEDLINE
DOCUMENT NUMBER: 91268059 PubMed ID: 1646817
TITLE: cAMP and cAMP-dependent protein kinase regulate the human heat shock protein 70 gene promoter activity.
AUTHOR: Choi H S; Li B; Lin Z; Huang E; Liu A Y
CORPORATE SOURCE: Department of Biological Sciences, Rutgers-State University of New Jersey, Piscataway 08855-1059.
SOURCE: JOURNAL OF BIOLOGICAL CHEMISTRY, (1991 Jun 25) 266 (18) 11858-65.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199107
ENTRY DATE: Entered STN: 19910811
Last Updated on STN: 19980206
Entered Medline: 19910724
AB The theme of this study is an evaluation of the involvement of cAMP and cAMP-dependent protein kinase (PKA) in the regulation of the human heat shock protein (hsp) 70 gene promoter. Expression of a highly specific protein inhibitor of PKA (pRSVPKI) inhibited the basal as well as heat- and cadmium-induced expression of the **cotransfected** pHBCAT, a human hsp 70 promoter-driven reporter gene; this inhibition was dependent on the amount of pRSVPKI used. The effect of an expression vector of the RI regulatory **subunit** of **PKA**, pMTREV, was similar to that of pRSVPKI; pMTREV inhibited both the basal as well as the heat-induced expression of pHBCAT. The specificity of effects of these expression vectors was demonstrated by the lack of effect of a mutant PKI

gene and by the unaffected expression of a reference gene (pRSV beta gal) under these conditions. Analysis of the effects of dibutyryl cAMP (1 mM), forskolin (10 microM), and 8-Br-cAMP (1 mM) on the transient expression of pHBCAT showed that these cAMP-elevating agents stimulated the hsp 70 promoter activity, whereas cAMP (1 mM) was without effect. Chloramphenicol acetyltransferase gene constructs with truncated or mutated hsp 70 promoter were used to define the cis-acting DNA element(s) that confer this cAMP stimulation; the heat induced (42 degrees C) expression was used as a control. Mutation of the adenovirus transcription factor element (pLSN-40/-26) greatly reduced the basal level of expression; forskolin had little or no effect on this adenovirus transcription factor-minus promoter, although the promoter activity was very heat inducible. The absence of a functional heat shock consensus element (HSE) in the construct pLSPNWT rendered the promoter heat insensitive; this construct was forskolin responsive although the magnitude of this stimulation was reduced when compared with that of a control construct with HSE. These results were corroborated by studies using consensus sequence of ATF (ATFE) and HSE as competitors to titrate our cellular factors that may interact with these elements. We showed that **cotransfection** with ATFE and HSE depressed the basal (37 degrees C) expression of pHBCAT by 25 and 60%, respectively. The heat-induced expression of pHBCAT was not significantly affected by the **cotransfection** of ATFE and was reduced by 60% when HSE was **cotransfected**. ATFE and HSE reduced the forskolin-induced pHBCAT expression by 70 and 40%, respectively. The implications of these findings as they relate to the action of cAMP and cAMP-dependent protein kinase in the control of heat shock gene expression are discussed.

L8 ANSWER 16 OF 17 MEDLINE DUPLICATE 14
ACCESSION NUMBER: 92123225 MEDLINE
DOCUMENT NUMBER: 92123225 PubMed ID: 1663213
TITLE: The protooncogene c-fos is induced by corticotropin-releasing factor and stimulates proopiomelanocortin gene transcription in pituitary cells.
AUTHOR: Boutillier A L; Sassone-Corsi P; Loeffler J P
CORPORATE SOURCE: Institut de Physiologie et de Chimie Biologique, URA 1446 du CNRS, Strasbourg, France.
CONTRACT NUMBER: NIHDK 27484 (NICHD)
SOURCE: MOLECULAR ENDOCRINOLOGY, (1991 Sep) 5 (9) 1301-10. Journal code: NGZ; 8801431. ISSN: 0888-8809.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199202
ENTRY DATE: Entered STN: 19920315
Last Updated on STN: 19960129
Entered Medline: 19920225

AB CRF is a potent hypophysiotropic factor which stimulates POMC-producing cells in both the intermediate and anterior pituitary. Although its secretagogue effects and its stimulatory action on POMC gene expression are well documented, the mechanisms by which CRF modulates gene regulation are poorly understood. In this study we have investigated the mechanisms by which CRF stimulates the immediate early gene c-fos. Studies were performed in the corticotroph-derived AtT20 cell line. We show that CRF induces a transient increase in c-fos mRNA levels. This induction is reduced by blockade of calcium entry and by calmodulin inhibitors, suggesting that the CRF-induced c-fos increase is mediated in part by the second messenger Ca2+ and the Ca2+/calmodulin kinase. When protein kinase-A (PKA) was inhibited by introduction of a mutated regulatory **subunit** of **PKA** that lacks cAMP-binding sites, the stimulation of c-fos mRNA by CRF was abolished. Taken together, these

results suggest that CRF activates the c-fos proto-oncogene via PKA and the Ca2+/calmodulin kinase. These results were confirmed and extended by gene transfer studies using chimera genes containing c-fos promoter sequences coupled to the chloramphenicol acetyl transferase reporter gene. This series of experiments shows that CRF stimulates c-fos transcription by mechanisms requiring PKA activation. Furthermore, **cotransfection** experiments with the POMC promoter linked to the chloramphenicol acetyl transferase reporter gene along with an expression vector coding for cFOS showed efficient stimulation of POMC gene transcription by cFOS. In summary, c-fos mRNA accumulation is an early genomic signal in pituitary cells in response to CRF, and cFOS may represent a signal controlling POMC gene expression.

L8 ANSWER 17 OF 17 MEDLINE
ACCESSION NUMBER: 91344227 MEDLINE
DOCUMENT NUMBER: 91344227 PubMed ID: 1652168
TITLE: Intracellular mechanisms of gonadotropin-stimulated gene expression in granulosa cells.
AUTHOR: Melner M H; Young S L
CORPORATE SOURCE: Division of Reproductive Biology, Oregon Regional Primate Research Center, Beaverton 97006.
CONTRACT NUMBER: DK-41035 (NIDDK)
RR-00163 (NCRR)
SOURCE: STEROIDS, (1991 May) 56 (5) 232-6.
Journal code: V10; 0404536. ISSN: 0039-128X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199109
ENTRY DATE: Entered STN: 19911013
Last Updated on STN: 19980206
Entered Medline: 19910924

AB Previous studies have shown that the gonadotropins follicle-stimulating hormone and luteinizing hormone stimulate proopiomelanocortin (POMC) promoter activity and mRNA levels in ovarian granulosa cells. The objective of these studies was to determine the role of cAMP-dependent protein kinases (pKA) in gonadotropin-stimulated gene expression. Primary cultures of rat granulosa cells were transfected with a gene construct consisting of the POMC promoter (-150 to +63; designated pOMC-CAT) fused to the chloramphenicol acetyltransferase (CAT) reporter gene either alone or **cotransfected** with an expression plasmid (designated mutant RI), which overexpresses a mutant form of the murine RI subunit incapable of binding cAMP and serving as an irreversible inhibitor of the catalytic **subunit** of **pKA**. Follicle-stimulating hormone or isoproterenol caused a significant stimulation of pOMC-CAT activity in transfected cells. **Cotransfection** of pOMC-CAT with mutant RI caused a significant inhibition of basal pOMC-CAT activity and abolished the gonadotropin stimulation. As a control, transfection of the SV-40 viral enhancer-promoter fused to CAT (pSV2-CAT) was unresponsive to follicle-stimulating hormone stimulation and **cotransfection** with mutant RI had no significant effect on pSV2-CAT activity. These studies suggest that gonadotropin regulation of the POMC promoter is mediated by pKA and that promoter activity is stringently controlled by pKA.

=> d his

(FILE 'HOME' ENTERED AT 15:38:38 ON 24 MAY 2001)

FILE 'MEDLINE, BIOSIS, BIOTECHNO' ENTERED AT 15:46:23 ON 24 MAY 2001
L1 588 S SUBUNIT OF PKA
L2 26 S L1 AND CRE
L3 11 DUP REM L2 (15 DUPLICATES REMOVED)
L4 19 S CRE-CREB
L5 10 DUP REM L4 (9 DUPLICATES REMOVED)

L6 47 S L[redacted] AND COTRANSFECT?
L7 41 S L[redacted] NOT L2
L8 17 DUP REM L7 (24 DUPLICATES REMOVED)
L9 1 S L8 AND (AMPLIF? OR FEEDBACK)

=> s coexpression of CREB

L10 4 COEXPRESSION OF CREB

=> dup rem l10

PROCESSING COMPLETED FOR L10
L11 2 DUP REM L10 (2 DUPLICATES REMOVED)

=> d ibib abs 1-2

L11 ANSWER 1 OF 2 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 1999323534 MEDLINE
DOCUMENT NUMBER: 99323534 PubMed ID: 10397405
TITLE: Cyclic AMP response element mediates dexamethasone induced suppression of prostaglandin H synthase-2 gene expression in human amnion derived WISH cells.
AUTHOR: Wang Z; Tai H H
CORPORATE SOURCE: Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky, Lexington 40536-0082, USA.
SOURCE: PROSTAGLANDINS LEUKOTRIENES AND ESSENTIAL FATTY ACIDS, (1999 Apr) 60 (4) 243-8.
Journal code: P04; 8802730. ISSN: 0952-3278.
PUB. COUNTRY: SCOTLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199908
ENTRY DATE: Entered STN: 19990827
Last Updated on STN: 19990827
Entered Medline: 19990819

AB A human PGHS-2 promoter fragment (300 BP) linked to the luciferase reporter was used to study the regulation of PGHS-2 gene expression in human amnion-derived WISH cells. A cyclic AMP (cAMP) response element (CRE) was found to be important in the induction of PGHS-2 gene expression. This was demonstrated by showing that **coexpression** of **CREB** stimulated native but not CRE mutant promoter and that IL-1beta and PMA induced less activity with the mutant promoter as compared to the native promoter. The effect of dexamethasone on IL-1beta and PMA induced promoter activities was further examined. IL-1beta or PMA induced activity was blocked by dexamethasone, whereas IL-1beta or PMA induced mutant activity was not responsive to dexamethasone. Direct activation of CRE by a cAMP elevating agent, isoproterenol, was found to be inhibited significantly dexamethasone. These results suggest that CRE may mediate the induction of PGHS-2 by IL-1beta and PMA as well as the suppression of expression by dexamethasone in amnion-derived cells.

L11 ANSWER 2 OF 2 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 97232267 MEDLINE
DOCUMENT NUMBER: 97232267 PubMed ID: 9077542
TITLE: Proliferation of hepatic stellate cells is inhibited by phosphorylation of CREB on serine 133.
AUTHOR: Houglum K; Lee K S; Chojkier M
CORPORATE SOURCE: Department of Medicine, University of California, San Diego
92161, USA.
CONTRACT NUMBER: DK-38652 (NIDDK)
DK-46971 (NIDDK)
GM-47165 (NIGMS)
+
SOURCE: JOURNAL OF CLINICAL INVESTIGATION, (1997 Mar 15) 99 (6) 1322-8.

Journal code: HS7; 7802877. ISSN 0021-9738.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199704
ENTRY DATE: Entered STN: 19970507
Last Updated on STN: 19980206
Entered Medline: 19970425

AB Proliferating, activated, hepatic stellate cells have a high level of collagen type I expression. Therefore, stellate cell proliferation is a critical step in hepatic fibrosis. Here we show that proliferation of activated primary rat stellate cells was blocked by elevation of cAMP with 8 Br-cAMP or isomethylbutyl xanthine, a phosphodiesterase inhibitor, and by stimulation of Ca2+ fluxes with the Ca2+ ionophore A-23187. Because phosphorylation of CREB on Ser133 is an important mediator of cAMP-protein kinase (PKA) and Ca2+-calmodulin kinase II (CAMK-II) activation, we tested whether CREB-PSer133 was essential for stellate cell quiescence. Nuclear extracts from quiescent, but not from activated, stellate cells contained CREB-PSer133. Moreover, the phosphorylation of CREB on Ser133 was stimulated in activated cells by inducing the activity of PKA or CAMK-II. In addition, **coexpression** of **CREB** and either a constitutively active PKA or a constitutively active CAMK-II inhibited the proliferation of activated stellate cells. In contrast, expression of CREB alone, PKA or CAMK-II alone, CREB-Ala 133 (which lacks the Ser133 phosphoacceptor) with PKA or CAMK-II, or CREB with inactive PKA or CAMK-II mutants did not affect stellate cell proliferation, suggesting that CREB-PSer133 is necessary for blocking the stellate cell cycle. Conversely, expression of a trans-dominant negative CREB-Ala 133 mutant (which competes with CREB/CREB-PSer133 for cognate DNA binding sites and presumably for protein interactions) induced a greater than fivefold entry into S-phase of quiescent stellate cells, compared with control cells expressing either beta-galactosidase or wt CREB, indicating that CREB-PSer133 may be indispensable for the quiescent stellate cell phenotype. This study suggests that PKA and CAMK-II play an essential role on stellate cell activation through the induction of CREB phosphorylation on Ser133, and provides potential approaches for the treatment of hepatic fibrogenesis in patients with chronic liver diseases.

```
=> s increased expression of creb

L12          3 INCREASED EXPRESSION OF CREB

=> dup rem l12

PROCESSING COMPLETED FOR L12
L13          1 DUP REM L12 (2 DUPLICATES REMOVED)

=> d ibib abs
```

L13 ANSWER 1 OF 1 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 96180950 MEDLINE
DOCUMENT NUMBER: 96180950 PubMed ID: 8601816
TITLE: Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus.
AUTHOR: Nibuya M; Nestler E J; Duman R S
CORPORATE SOURCE: Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06508, USA.

CONTRACT NUMBER: [redacted]1 MH25642 (NIMH)
[redacted]5481 (NIMH)
MH53199 (NIMH)
SOURCE: JOURNAL OF NEUROSCIENCE, (1996 Apr 1) 16 (7) 2365-72.
Journal code: JDF; 8102140. ISSN: 0270-6474.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199605
ENTRY DATE: Entered STN: 19960517
Last Updated on STN: 20000303
Entered Medline: 19960509

AB The present study demonstrates that chronic, but not acute, adminstration of several different classes of antidepressants, including serotonin- and norepinephrine-selective reuptake inhibitors, increases the expression of cAMP response element binding protein (CREB) mRNA in rat hippocampus. In contrast, chronic administration of several nonantidepressant psychotropic drugs did not influence expression of CREB mRNA, demonstrating the pharmacological specificity of this effect. In situ hybridization analysis demonstrates that antidepressant administration increases expression of CREB mRNA in CA1 and CA3 pyramidal and dentate gyrus granule cell layers of the hippocampus. In addition, levels of CRE immunoreactivity and of CRE binding activity were increased by chronic antidepressant administration, which indicates that expression and function of CREB protein are increased along with its mRNA. Chronic administration of the phosphodiesterase (PDE) inhibitors rolipram or papaverine also **increased expression** of **CREB** mRNA in hippocampus, demonstrating a role for the cAMP cascade. Moreover, coadministration of rolipram with imipramine resulted in a more rapid induction of CREB than with either treatment alone. Increased expression and function of CREB suggest that specific target genes may be regulated by these treatments. We have found that levels of brain-derived neurotrophic factor (BDNF) and trkB mRNA are also increased by administration of antidepressants or PDE inhibitors. These findings indicate that upregulation of CREB is a common action of chronic antidepressant treatments that may lead to regulation of specific target genes, such as BDNF and trkB, and to the long-term effects of these treatments on brain function.

=> expression of creb

EXPRESSION IS NOT A RECOGNIZED COMMAND
The previous command name entered was not recognized by the system.
For a list of commands available to you in the current file, enter
"HELP COMMANDS" at an arrow prompt (=>).

=> s expression of creb

L14 96 EXPRESSION OF CREB

=> s l14 and coexpression

L15 5 L14 AND COEXPRESSION

=> dup rem l15

PROCESSING COMPLETED FOR L15
L16 2 DUP REM L15 (3 DUPLICATES REMOVED)

=> d ibib abs 1-2

L16 ANSWER 1 OF 2 [redacted]LINE [redacted] DUPLICATE 1
ACCESSION NUMBER: [redacted]0092329 MEDLINE
DOCUMENT NUMBER: 20092329 PubMed ID: 10628748
TITLE: Synergistic activation of the inhibin alpha-promoter by steroidogenic factor-1 and cyclic adenosine 3',5'-monophosphate.
AUTHOR: Ito M; Park Y; Weck J; Mayo K E; Jameson J L
CORPORATE SOURCE: Division of Endocrinology, Metabolism, and Molecular Medicine, Northwestern University Medical School, Chicago, Illinois 60611, USA.
CONTRACT NUMBER: PO1 HD-21921 (NICHD)
U54-HD-29164 (NICHD)
SOURCE: MOLECULAR ENDOCRINOLOGY, (2000 Jan) 14 (1) 66-81.
Journal code: NGZ; 8801431. ISSN: 0888-8809.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200001
ENTRY DATE: Entered STN: 20000204
Last Updated on STN: 20000204
Entered Medline: 20000124

AB The inhibin alpha-subunit gene is expressed in the ovary, testis, adrenal, and pituitary. Because this pattern of expression corresponds to that of the orphan nuclear receptor, steroidogenic factor-1 (SF-1), we hypothesized that the inhibin alpha promoter might be regulated by SF-1. Expression of exogenous SF-1, in an SF-1 deficient cell line, caused modest stimulation of the inhibin alpha promoter. However, activation of the cAMP pathway, which is known to regulate inhibin alpha expression, greatly enhanced the actions of SF-1. **Coexpression** of SF-1 with the catalytic subunit of cAMP-dependent protein kinase A caused greater than 250-fold stimulation, whereas only 4- or 7-fold stimulation was seen by the SF-1 or protein kinase A pathway alone. Synergistic stimulation by SF-1 and the cAMP pathway was also seen in GRMO2 granulosa cells, which express endogenous SF-1. Deletion and site-directed mutagenesis localized a novel SF-1 regulatory element (TCA GGGCCA; -137 to -129) adjacent to a variant cAMP-response element (CRE; -120 to -114). The synergistic property of SF-1 and cAMP stimulation was inherent within this composite inhibin alpha fragment (-146 and -112), as it was transferable to heterologous promoters. Mutations in either the CRE or the SF-1 regulatory element completely eliminated synergistic activation by these pathways. The binding of SF-1 and CRE binding protein (CREB) to the inhibin alpha regulatory elements was relatively weak in gel mobility shift assays, consistent with their deviation from consensus binding sites. However, SF-1 was found to interact with CREB using an assay in which epitope-tagged SF-1 was expressed in cells and used to pull down in vitro translated CREB. **Expression** of **CREB** binding protein (CBP), a coactivator that interacts with SF-1 and CREB, further enhanced transcription by these pathways. Stimulation by the SF-1 and cAMP pathways was associated with increased histone H4 acetylation, suggesting that chromatin remodeling accompanies their actions. We propose a model in which direct interactions of SF-1, CREB, and associated coactivators like CBP induce strongly cooperative transactivation by pathways that individually have relatively weak effects on transcription.

L16 ANSWER 2 OF 2 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 97232267 MEDLINE
DOCUMENT NUMBER: 97232267 PubMed ID: 9077542
TITLE: Proliferation of hepatic stellate cells is inhibited by phosphorylation of CREB on serine 133.
AUTHOR: Houglum K; Lee K S; Chojkier M
CORPORATE SOURCE: Department of Medicine, University of California, San Diego 92161, USA.
CONTRACT NUMBER: DK-38652 (NIDDK)
DK-46971 (NIDDK)

[redacted]-47165 (NIGMS)

SOURCE: JOURNAL OF CLINICAL INVESTIGATION, (1997 Mar 15) 99 (6) 1322-8.
Journal code: HS7; 7802877. ISSN: 0021-9738.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199704
ENTRY DATE: Entered STN: 19970507
Last Updated on STN: 19980206
Entered Medline: 19970425

AB Proliferating, activated, hepatic stellate cells have a high level of collagen type I expression. Therefore, stellate cell proliferation is a critical step in hepatic fibrosis. Here we show that proliferation of activated primary rat stellate cells was blocked by elevation of cAMP with 8 Br-cAMP or isomethylbutyl xanthine, a phosphodiesterase inhibitor, and by stimulation of Ca2+ fluxes with the Ca2+ ionophore A-23187. Because phosphorylation of CREB on Ser133 is an important mediator of cAMP-protein kinase (PKA) and Ca2+-calmodulin kinase II (CAMK-II) activation, we tested whether CREB-PSer133 was essential for stellate cell quiescence. Nuclear extracts from quiescent, but not from activated, stellate cells contained CREB-PSer133. Moreover, the phosphorylation of CREB on Ser133 was stimulated in activated cells by inducing the activity of PKA or CAMK-II. In addition, **coexpression** of CREB and either a constitutively active PKA or a constitutively active CAMK-II inhibited the proliferation of activated stellate cells. In contrast, **expression** of **CREB** alone, PKA or CAMK-II alone, CREB-Ala 133 (which lacks the Ser133 phosphoacceptor) with PKA or CAMK-II, or CREB with inactive PKA or CAMK-II mutants did not affect stellate cell proliferation, suggesting that CREB-PSer133 is necessary for blocking the stellate cell cycle. Conversely, expression of a trans-dominant negative CREB-Ala 133 mutant (which competes with CREB/CREB-PSer133 for cognate DNA binding sites and presumably for protein interactions) induced a greater than fivefold entry into S-phase of quiescent stellate cells, compared with control cells expressing either beta-galactosidase or wt CREB, indicating that CREB-PSer133 may be indispensable for the quiescent stellate cell phenotype. This study suggests that PKA and CAMK-II play an essential role on stellate cell activation through the induction of CREB phosphorylation on Ser133, and provides potential approaches for the treatment of hepatic fibrogenesis in patients with chronic liver diseases.

=> d his

(FILE 'HOME' ENTERED AT 15:38:38 ON 24 MAY 2001)

FILE 'MEDLINE, BIOSIS, BIOTECHNO' ENTERED AT 15:46:23 ON 24 MAY 2001

L1	588	S SUBUNIT OF PKA
L2	26	S L1 AND CRE
L3	11	DUP REM L2 (15 DUPLICATES REMOVED)
L4	19	S CRE-CREB
L5	10	DUP REM L4 (9 DUPLICATES REMOVED)
L6	47	S L1 AND COTRANSFECT?
L7	41	S L6 NOT L2
L8	17	DUP REM L7 (24 DUPLICATES REMOVED)
L9	1	S L8 AND (AMPLIF? OR FEEDBACK)
L10	4	S COEXPRESSION OF CREB
L11	2	DUP REM L10 (2 DUPLICATES REMOVED)
L12	3	S INCREASED EXPRESSION OF CREB
L13	1	DUP REM L12 (2 DUPLICATES REMOVED)
L14	96	S EXPRESSION OF CREB

L15 5 S L1 AND COEXPRESSION
L16 2 DUP [redacted] L15 (3 DUPLICATES REMOVED)

=> s l14 and (amplif? or feedback or cooperative)

L17 11 L14 AND (AMPLIF? OR FEEDBACK OR COOPERATIVE)

=> dup rem l17

PROCESSING COMPLETED FOR L17
L18 5 DUP REM L17 (6 DUPLICATES REMOVED)

=> d ibib abs 1-5

L18 ANSWER 1 OF 5 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 2000092329 MEDLINE
DOCUMENT NUMBER: 20092329 PubMed ID: 10628748
TITLE: Synergistic activation of the inhibin alpha-promoter by steroidogenic factor-1 and cyclic adenosine 3',5'-monophosphate.
AUTHOR: Ito M; Park Y; Weck J; Mayo K E; Jameson J L
CORPORATE SOURCE: Division of Endocrinology, Metabolism, and Molecular Medicine, Northwestern University Medical School, Chicago, Illinois 60611, USA.
CONTRACT NUMBER: PO1 HD-21921 (NICHD)
U54-HD-29164 (NICHD)
SOURCE: MOLECULAR ENDOCRINOLOGY, (2000 Jan) 14 (1) 66-81.
Journal code: NGZ; 8801431. ISSN: 0888-8809.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200001
ENTRY DATE: Entered STN: 20000204
Last Updated on STN: 20000204
Entered Medline: 20000124

AB The inhibin alpha-subunit gene is expressed in the ovary, testis, adrenal, and pituitary. Because this pattern of expression corresponds to that of the orphan nuclear receptor, steroidogenic factor-1 (SF-1), we hypothesized that the inhibin alpha promoter might be regulated by SF-1. Expression of exogenous SF-1, in an SF-1 deficient cell line, caused modest stimulation of the inhibin alpha promoter. However, activation of the cAMP pathway, which is known to regulate inhibin alpha expression, greatly enhanced the actions of SF-1. Coexpression of SF-1 with the catalytic subunit of cAMP-dependent protein kinase A caused greater than 250-fold stimulation, whereas only 4- or 7-fold stimulation was seen by the SF-1 or protein kinase A pathway alone. Synergistic stimulation by SF-1 and the cAMP pathway was also seen in GRMO2 granulosa cells, which express endogenous SF-1. Deletion and site-directed mutagenesis localized a novel SF-1 regulatory element (TCA GGGCCA; -137 to -129) adjacent to a variant cAMP-response element (CRE; -120 to -114). The synergistic property of SF-1 and cAMP stimulation was inherent within this composite inhibin alpha fragment (-146 and -112), as it was transferable to heterologous promoters. Mutations in either the CRE or the SF-1 regulatory element completely eliminated synergistic activation by these pathways. The binding of SF-1 and CRE binding protein (CREB) to the inhibin alpha regulatory elements was relatively weak in gel mobility shift assays, consistent with their deviation from consensus binding sites. However, SF-1 was found to interact with CREB using an assay in which epitope-tagged SF-1 was expressed in cells and used to pull down in vitro translated CREB. **Expression** of **CREB** binding protein (CBP), a coactivator that interacts with SF-1 and CREB, further enhanced transcription by these pathways. Stimulation by the SF-1 and cAMP pathways was associated with increased histone H4 acetylation, suggesting that chromatin remodeling accompanies their actions. We propose a model in

which direct interactions of SF-1, CREB, and associated coactivators like CBP induce strongly **cooperative** transactivation pathways that individually have relatively weak effects on transcription.

L18 ANSWER 2 OF 5 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 1999021198 MEDLINE
DOCUMENT NUMBER: 99021198 PubMed ID: 9806361
TITLE: Inducible cAMP early repressor ICER down-regulation of CREB gene expression in Sertoli cells.
AUTHOR: Walker W H; Daniel P B; Habener J F
CORPORATE SOURCE: Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston 02114, USA.
CONTRACT NUMBER: DK25532 (NIDDK)
SOURCE: MOLECULAR AND CELLULAR ENDOCRINOLOGY, (1998 Aug 25) 143 (1-2) 167-78.
Journal code: E69; 7500844. ISSN: 0303-7207.
PUB. COUNTRY: Ireland
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199901
ENTRY DATE: Entered STN: 19990115
Last Updated on STN: 19990115
Entered Medline: 19990105

AB The cAMP response element binding protein (CREB) and the cAMP-responsive element modulator (CREM) are cyclically expressed in the seminiferous tubules during spermatogenesis. In the somatic Sertoli cells, which are the major supporters of germ cell development in the seminiferous tubules, the **expression** of **CREB** is cyclical and appears to be regulated by the levels of cAMP produced in response to the pituitary derived follicle-stimulating hormone FSH. Cyclic AMP response elements (CREs) located in the promoter of the CREB gene were shown earlier to be implicated in an autopositive **feedback** loop that up-regulates the **expression** of **CREB**. Here we show that in Sertoli cells FSH-mediated induction of the CREM repressor isoform, ICER (inducible cAMP early repressor) is correlated with the inhibition and delay of CREB gene expression in the seminiferous tubules. ICER binds to the two CREs located in the promoter of the CREB gene and in transient transfection assays of Sertoli cells, ICER expression vectors down-regulate transcription of a reporter gene driven by the CREB gene promoter. In addition, analyses of ICER and CREB gene expression in isolated segments of rat seminiferous tubules reveals stage-specific and cycle-dependent expression of ICER. The periods of enhanced expression of ICER correspond to the stages of spermatogenesis with the lowest levels of CREB expression. We suggest that the expression of ICER in Sertoli cells may contribute to the periodic repression of CREB gene expression during the repeated 12-day cycles of spermatogenesis, and may be required to reset the levels of activator CREB prior to the initiation of each new cycle of spermatogenesis.

L18 ANSWER 3 OF 5 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 1998260039 MEDLINE
DOCUMENT NUMBER: 98260039 PubMed ID: 9597751
TITLE: Coupling gene expression to cAMP signalling: role of CREB and CREM.
AUTHOR: Sassone-Corsi P
CORPORATE SOURCE: Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, C.U. de Strasbourg, France.
SOURCE: INTERNATIONAL JOURNAL OF BIOCHEMISTRY AND CELL BIOLOGY, (1998 Jan) 30 (1) 27-38. Ref: 57
Journal code: CDK; 9508482. ISSN: 1357-2725.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)

LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199807
ENTRY DATE: Entered STN: 19980723
Last Updated on STN: 19980723
Entered Medline: 19980715

AB Several endocrine and neuronal functions are governed by the cAMP-dependent pathway. Transcriptional regulation upon stimulation of this pathway is mediated by a family of cAMP-responsive nuclear factors. This family consists of a large number of members, which may act as activators or repressors. These factors contain the basic domain/leucine zipper motifs and bind as dimers to cAMP-response elements (CRE). CRE-binding protein (CREBs) function is modulated by phosphorylation by several kinases. Direct activation of gene **expression** by **CREB** requires phosphorylation by the cAMP-dependent PKA to serine 133. Among the repressors, ICER (Inducible cAMP Early Repressor) deserves special mention. ICER is generated from an alternative CREM promoter and is the only inducible CRE-binding protein. ICER negatively autoregulates the alternative promoter, generating a **feedback** loop. ICER expression is tissue specific and developmentally regulated. The kinetics of ICER expression are characteristic of an early response gene. CREM plays a key physiological and developmental role within the hypothalamic-pituitary-gonadal axis. The transcriptional activator CREM is highly expressed in postmeiotic cells. The role of CREM in spermiogenesis was addressed using CREM knock-out mice. Spermatogenesis stops at the first step of spermiogenesis in the mutants and there is a significant increase in apoptotic germ cells. This phenotype is reminiscent of cases of human infertility. ICER is regulated in a circadian manner in the pineal gland, the site of the hormone melatonin production. This night-day oscillation is driven by the endogenous clock (located in the suprachiasmatic nucleus). The synthesis of melatonin is regulated by a rate-limiting enzyme, serotonin N-acetyltransferase (NAT). Analysis of the CREM-null mice and of the promoter of the NAT gene revealed that ICER controls the amplitude and rhythmicity of NAT, and thus the oscillation in the hormonal synthesis of melatonin.

L18 ANSWER 4 OF 5 MEDLINE
ACCESSION NUMBER: 97381623 MEDLINE
DOCUMENT NUMBER: 97381623 PubMed ID: 9238850
TITLE: Coupling signalling pathways to transcriptional control: nuclear factors responsive to cAMP.
AUTHOR: Tamai K T; Monaco L; Nantel F; Zazopoulos E; Sassone-Corsi P
CORPORATE SOURCE: Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, Strasbourg, France.
SOURCE: RECENT PROGRESS IN HORMONE RESEARCH, (1997) 52 121-39; discussion 139-40. Ref: 93
Journal code: R1D; 0404471. ISSN: 0079-9963.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, ACADEMIC)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199708
ENTRY DATE: Entered STN: 19970908
Last Updated on STN: 19980206
Entered Medline: 19970826

AB Several endocrine and neuronal functions are governed by the cAMP-dependent signalling pathway. In eukaryotes, transcriptional regulation upon stimulation of the adenylyl cyclase signalling pathway is mediated by a family of cAMP-responsive nuclear factors. This family consists of a large number of members that may act as activators or repressors. These factors contain the basic domain/ leucine zipper motifs and bind as dimers to cAMP-response elements (CRE). The function of

CRE-binding proteins (CREBs) is modulated by phosphorylation by several kinases. Direct activation of gene **expression** by **CREB** requires phosphorylation by the cAMP-dependent protein kinase A to the serine-133 residue. Among the repressors, ICER (Inducible cAMP Early Repressor) deserves special mention. ICER is generated from an alternative CREM promoter and constitutes the only inducible cAMP-responsive element binding protein. Furthermore, ICER negatively autoregulates the alternative promoter, thus generating a **feedback** loop. In contrast to the other members of the CRE-binding protein family, ICER expression is tissue specific and developmentally regulated. The kinetics of ICER expression are characteristic of an early response gene. Our results indicate that CREM plays a key physiological and developmental role within the hypothalamic-pituitary-gonadal axis. We have previously shown that the transcriptional activator CREM is highly expressed in postmeiotic cells. Spermiogenesis is a complex process by which postmeiotic male germ cells differentiate into mature spermatozoa. This process involves remarkable structural and biochemical changes that are under the hormonal control of the hypothalamic-pituitary axis. We have addressed the specific role of CREM in spermiogenesis using CREM-mutant mice generated by homologous recombination. Analysis of the seminiferous epithelium from mutant male mice reveals that spermatogenesis stops at the first step of spermiogenesis. Late spermatids are completely absent, while there is a significant increase in apoptotic germ cells. A series of postmeiotic germ cell-specific genes are not expressed. Mutant male mice completely lack spermatozoa. This phenotype is reminiscent of cases of human infertility. We have shown that ICER is regulated in a circadian manner in the pineal gland, the site of the hormone melatonin production. This night-day oscillation is driven by the endogenous clock (located in the suprachiasmatic nucleus, SCN). The synthesis of melatonin is regulated by a rate-limiting enzyme, the serotonin N-acetyltransferase (NAT). By using the CREM-deficient mice and by analysis of the regulatory region of the gene encoding the serotonin NAT, we have established that ICER is responsible for the amplitude and rhythmicity of NAT and thus for the oscillation in the hormonal synthesis of melatonin.

L18 ANSWER 5 OF 5 MEDLINE DUPLICATE 4
ACCESSION NUMBER: 96028342 MEDLINE
DOCUMENT NUMBER: 96028342 PubMed ID: 7551568
TITLE: Serine 133-phosphorylated CREB induces transcription via a **cooperative** mechanism that may confer specificity to neurotrophin signals.
AUTHOR: Bonni A; Ginty D D; Dudek H; Greenberg M E
CORPORATE SOURCE: Program in Neuroscience, Harvard Medical School, Boston, Massachusetts 02115, USA.
CONTRACT NUMBER: RO1 CA43855 (NCI)
SOURCE: MOLECULAR AND CELLULAR NEUROSCIENCES, (1995 Apr) 6 (2) 168-83.
Journal code: B1D; 9100095. ISSN: 1044-7431.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199511
ENTRY DATE: Entered STN: 19951227
Last Updated on STN: 19970203
Entered Medline: 19951103

AB A mechanism has been characterized by which the transcription factor CREB regulates neurotrophin-induced gene **expression**. Whereas **CREB** can mediate calcium- or cyclic AMP-induced c-fos transcription independently of other promoter-bound transcription factors, CREB mediates NGF induction of c-fos transcription via a novel mechanism that appears to require a **cooperative** interaction with another transcription factor, the serum response factor. A similar transcriptional

mechanism may explain how neurotrophins and growth factors induce distinct subsets of delayed response genes. Neurotrophins induce the phosphorylation of CREB at a key regulatory site, Serine 133, with prolonged kinetics that are distinct from the transient kinetics of CREB phosphorylation elicited by growth factors. These results indicate that CREB is a versatile transcription factor that activates transcription via distinct mechanisms in a stimulus-specific manner. In addition, by selectively activating delayed response genes, CREB may confer specificity to neurotrophin signals that promote the survival and differentiation of neurons.

=> log y

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	65.82	67.77

STN INTERNATIONAL LOGOFF AT 16:24:38 ON 24 MAY 2001

L13 ANSWER 10 OF 17 MEDLINE
ACCESSION NUMBER: 97155512 MEDLINE
DOCUMENT NUMBER: 97155512
TITLE: Genetically modified Escherichia coli for colorimetric detection of inorganic and organic Hg compounds.
AUTHOR: Klein J; Altenbuchner J; Mattes R
CORPORATE SOURCE: Institute of Industrial Genetics, University of Stuttgart, Germany.
SOURCE: EXS, (1997) 80 133-51.
Journal code: BFZ.
PUB. COUNTRY: Switzerland
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199704
ENTRY WEEK: 19970404

AB A sensitive colorimetric bacterial system was developed for the detection of Hg(II) and organomercury compounds. The bioactive species, a recombinant Escherichia coli, produces proportionally elevated levels of the enzyme beta-galactosidase with increasing amounts of Hg. This is due to a **reporter** plasmid which carriers a Hg(II)-inducible promoter (mer promoter) from the Hg resistance transposon Tn501 regulating the transcription of a promoterless lacZ gene. Additionally, a pMB1 origin of replication without the natural RNA polymerase start site is fused downstream of the mer promoter leading to a Hg(II)-inducible plasmid replication, which results in an improved **signal**-to-**noise** ratio. To enhance the sensitivity of this cellular biosensor, the transport proteins for Hg(II) uptake are constitutively produced by a helper plasmid. To enable the detection of organically bound Hg, the Streptomyces lividans organomercurical lyase, an enzyme which catalyses the cleavage of C-Hg-bonds of organomercurial compounds, is also provided by the helper plasmid. Hg(II) and phenylmercuric acetate (PMA) concentrations as low as 5 x 10(-10) M (0.1 ppb) may be detected within a few minutes.

L13 ANSWER 6 OF 17 MEDLINE
ACCESSION NUMBER: 1998348965 MEDLINE
DOCUMENT NUMBER: 98348965
TITLE: Comparison of mutant forms of the green fluorescent protein
as expression markers in Chinese hamster ovary (CHO) and Saccharomyces cerevisiae cells.
AUTHOR: Natarajan A; Subramanian S; Srienc F
CORPORATE SOURCE: Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, USA.
SOURCE: JOURNAL OF BIOTECHNOLOGY, (1998 Jun 11) 62 (1) 29-45.
Journal code: AL6. ISSN: 0168-1656.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199810
ENTRY WEEK: 19981004
AB Several green fluorescent protein (Gfp) mutants with increased cellular fluorescence compared to the wildtype protein have recently been generated. We have expressed and compared wildtype Gfp and mutants S65T, F100S/ M154T/V164A, F64L/S65T, and S65A/V68L/S72A under identical growth conditions in CHO and Saccharomyces cerevisiae cells. The results suggest that the last two Gfp mutants are the best candidates as **reporter** proteins, and they provide a high **signal**-to-**noise** ratio in both systems. Single gene copy expression of these mutant forms is easily detectable over background autofluorescence. All Gfps are highly stable within cells, with an estimated 1/2-life between 7 h (wildtype) and 70 h (F100S/M154T/V164A) in S. cerevisiae cells. Although this limits their use in examining rapid cellular events without further modification, Gfp is expected to be a useful marker for monitoring the physiological

L13 ANSWER 5 OF 17 MEDLINE
ACCESSION NUMBER: 1998447473 MEDLINE
DOCUMENT NUMBER: 98447473
TITLE: A highly sensitive and specific assay using a novel human growth hormone cDNA **reporter** gene regulated by the human interleukin-4 inducible germline epsilon transcript promoter.
AUTHOR: Jenh C H; Cox M A; Lundell D; Narula S K; Zavodny P J
CORPORATE SOURCE: Department of Immunology, Schering-Plough Research Institute, Kenilworth, NJ 07033, USA.. chung-her.jenh@spcorp.com
SOURCE: JOURNAL OF IMMUNOLOGICAL METHODS, (1998 Aug 1) 217 (1-2) 87-95.
Journal code: IFE. ISSN: 0022-1759.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 199901
ENTRY WEEK: 19990104

AB We have successfully developed a highly sensitive and specific assay system for human interleukin-4 (IL-4) regulated gene expression. It is based on a human Jijoye cell line with the germline epsilon transcript promoter joined to the human growth hormone (hGH) cDNA. The germline epsilon transcript promoter is responsive to IL-4 and involved in immunoglobulin heavy chain class switching. We cloned hGH complementary DNA (cDNA) as the **reporter** gene instead of using conventional hGH genomic DNA which failed to generate any IL-4 inducible clone in human Jijoye cells. The two IL-4 inducible cell lines with the hGH cDNA **reporter** show high **signal/noise** ratio for IL-4-mediated induction (60-90 fold). The response to IL-4 is dose-dependent with ED50 of 10 pM. As expected, there is no response to other human cytokines and growth factors, as well as mouse IL-4. The mutant hIL-4 antagonist hIL-4.Y124D inhibits the induction mediated by native hIL-4. These IL-4 inducible cell lines provide a sensitive, specific assay system to study IL-4-regulated gene expression, and in particular the regulation of the germline epsilon promoter.

L13 ANSWER 4 OF 17 MEDLINE
ACCESSION NUMBER: 1999045819 MEDLINE
DOCUMENT NUMBER: 99045819
TITLE: Development of a green fluorescent protein **reporter** for a yeast genotoxicity biosensor.
AUTHOR: Billinton N; Barker M G; Michel C E; Knight A W; Heyer W D;
Goddard N J; Fielden P R; Walmsley R M
CORPORATE SOURCE: Department of Biomolecular Sciences, UMIST, Manchester, UK.
SOURCE: BIOSENSORS AND BIOELECTRONICS, (1998 Oct 1) 13 (7-8) 831-8.
Journal code: AKA. ISSN: 0956-5663.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199903
ENTRY WEEK: 19990304
AB A **reporter** system, constructed for a laboratory screen for new genes involved in DNA repair in the brewer's yeast Saccharomyces cerevisiae, has been developed for use in a genotoxicity biosensor. The strain produces green fluorescent protein (yEGFP) when DNA damage has occurred. yEGFP is codon optimised for yeasts. The **reporter** does not respond to chemicals which delay mitosis, and responds appropriately to the genetic regulation of DNA repair. Data is presented which demonstrate strain improvements appropriate to biosensor technology: improved **signal** to **noise** ratio, ease of data collection and uncomp

L8 ANSWER 2 OF 12 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 1999196682 MEDLINE
DOCUMENT NUMBER: 99196682
TITLE: Viral protease assay based on GAL4 inactivation is applicable to **high-throughput** screening in mammalian cells.
AUTHOR: Lawler J F Jr; Snyder S H
CORPORATE SOURCE: Departments of Neuroscience, Pharmacology and Molecular Sciences, and Psychiatry, Johns Hopkins University School of Medicine, 725 N., Wolfe Street, Baltimore, Maryland 21205-2185, USA.
CONTRACT NUMBER: MH-18501 (NIMH)
DA-00674 (NIDA)
GM-07309 (NIGMS)
SOURCE: ANALYTICAL BIOCHEMISTRY, (1999 Apr 10) 269 (1) 133-8.
Journal code: 4NK. ISSN: 0003-2697.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199908
AB We present an assay for viral proteases that relies on the proteolytic cleavage of substrate leading to the dissociation of the yeast transcription factor GAL4. A consensus substrate for the cytomegalovirus protease is fused between the DNA binding and transactivating domains of GAL4. Proteolysis inactivates the transcription factor which drives a luciferase reporter system. The assay is performed in mammalian cells, has a robust **signal**-to-**noise** ratio, and assesses proteolysis in a physiologic context. A unique feature of the assay is its ability to detect inhibitors of viral replication that act on viral targets other than the protease. Copyright 1999 Academic Press.

4 ANSWER 2 OF 12 BIOTECHDS COPYRIGHT 2000 DERWENT INFORMATION LTD
ACCESSION NUMBER: 1998-07622 BIOTECHDS
TITLE: Detecting compounds that mimic or modulate the effects of ob-protein;
cachexia, obesity, anorexia and diabetes drug screening
AUTHOR: Beeley L J
PATENT ASSIGNEE: SK-Beecham
LOCATION: Brentford, UK.
PATENT INFO: WO 98200158 14 May 1998
APPLICATION INFO: WO 1997-GB2988 30 Oct 1997
PRIORITY INFO: GB 1996-22850 1 Nov 1996; GB 1996-22866 1 Nov 1996
DOCUMENT TYPE: Patent
LANGUAGE: English
OTHER SOURCE: WPI: 1998-286968 [25]
AN 1998-07622 BIOTECHDS
AB New methods for detecting a compound that mimics or potentiates or inhibits the physiological effect of the ob protein (or leptin) comprise assessing the effect of the compound on: an ob-protein activated signal transducer and activator of transcription (STAT) DNA response element coupled to a **reporter** gene; and the response provided by ob-protein on an ob-protein activated STAT DNA response element coupled to a **reporter** gene. The response element and the **reporter** are expressed in an ob-protein responsive cell line selected from hypothalamic, pheochromocytoma, hematopoietic, pancreatic, liver, preadipocyte, skeletal muscle or ovarian derived cell lines, alternatively, they are expressed in an engineered cell line which is also transfected with a polypeptide capable of stimulating an ob-protein activated STAT DNA response element and containing the appropriate STAT proteins. The method is useful in **high throughput** assays for compounds to treat weight, energy balance, hematopoietic, fertility and other disorders involving ob protein, especially disorders related to obesity, anorexia, cachexia and diabetes. (20pp)

L4 ANSWER 4 OF 12 BIOTECHDS COPYRIGHT 2000 DERWENT INFORMATION LTD
ACCESSION NUMBER: 1999-08516 BIOTECHDS
TITLE: Development of a green fluorescent protein **reporter** for a yeast genotoxicity biosensor;
DNA damage-stimulated yeast enhanced green fluorescent protein expression in Saccharomyces cerevisiae, useful for
drug screening, detecting DNA repair gene and environmental stress
AUTHOR: Billinton N; Barker M G; Michel C E; Knight A W; Heyer W D; Goddard N J; Fielden P R; *Walmsley R M
CORPORATE SOURCE: Univ.Manchester-Inst.Sci.Technol.
LOCATION: Department of Biomolecular Sciences and Department of Instrumentation and Analytical Science, UMIST, Manchester M60
1QD, UK.
Email: walmsley@umist.ac.uk
SOURCE: Biosensors Bioelectron.; (1998) 13, 7-8, 831-38
CODEN: BBIOE4
ISSN: 0956-5663
DOCUMENT TYPE: Journal
LANGUAGE: English
AN 1999-08516 BIOTECHDS
AB A **reporter** system was developed and described for use in a genotoxicity sensor to screen for new genes involved in DNA repair in Saccharomyces cerevisiae. If DNA damage has occurred, the strain produces yeast enhanced green fluorescent protein (GFP) by expression of plasmid pWDH443. The GFP was codon optimized for yeasts. The **reporter** effectively responds to the genetic regulation of DNA repair and does not respond to chemicals which delay mitosis. The improved strain demonstrates improved signal to noise ratio, convenient data collection and uncomplicated material handling. The combination of inducible promoter and GFP **reporter** allows environmental stress monitoring and drug screening by simple and continuous, **high throughput** biosensor technology. The generic technology of the **reporter** could be tailored to more specific sensing applications. (26 ref)

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FILE 'HOME' ENTERED AT 17:28:01 ON 24 MAY 2001

=> file medline biosis

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	0.15	0.15

FILE 'MEDLINE' ENTERED AT 17:28:10 ON 24 MAY 2001

FILE 'BIOSIS' ENTERED AT 17:28:10 ON 24 MAY 2001
COPYRIGHT (C) 2001 BIOSIS(R)

=> s (Francis j s)/au

L1 26 (FRANCIS J S)/AU

=> dup rem l1

PROCESSING COMPLETED FOR L1
L2 23 DUP REM L1 (3 DUPLICATES REMOVED)

=> s l2 and creb

L3 1 L2 AND CREB

=> s l2 and cre

L4 1 L2 AND CRE

=> d ibib abs 1-23

L4 ANSWER 1 OF 1 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 2001:60444 BIOSIS
DOCUMENT NUMBER: PREV200100060444
TITLE: Inhibition of **CRE**-mediated gene expression by CREB within the context of an apoptotic stimulus.
AUTHOR(S): **Francis, J. S. (1)**; During, M. J.
CORPORATE SOURCE: (1) Thomas Jefferson Univ, Philadelphia, PA USA
SOURCE: Society for Neuroscience Abstracts, (2000) Vol. 26, No. 1-2, pp. Abstract No.-49.16. print.
Meeting Info.: 30th Annual Meeting of the Society of Neuroscience New Orleans, LA, USA November 04-09, 2000 Society for Neuroscience
. ISSN: 0190-5295.
DOCUMENT TYPE: Conference
LANGUAGE: English
SUMMARY LANGUAGE: English
AB Activation of the cAMP response element binding protein (CREB) is a feature of the cellular response to insults that are associated with apoptosis. CREB is thought to activate target gene expression primarily through an interaction with the cAMP response element (**CRE**) found within the promoter of these genes. An in vitro model of stress-activated cellular signaling was used to investigate the activity of **CRE**-mediated transcriptional activity within the context of an apoptotic insult. Mouse C17.2 neural precursor cells were transfected with a **CRE**-containing luciferase reporter cassette prior to exposure to okadiac acid, in order to investigate the role of cAMP-mediated transcriptional activation within an apoptotic context.

Exposure of cell[redacted]o okadiac acid resulted in a 2[redacted]old induction of luciferase expression that was detectable as early as 15 minutes after exposure, and maximal after 6 hours. Cotransfection of a **CRE**-CREB cassette significantly reduced okadiac acid- associated induction of luciferase expression. This apparent inhibition by **CRE**-CREB was reversed by the presence of constitutively expressed dominant-negative CREB mutant (A-CREB), suggesting that this phenomenon is a direct consequence of CREB activity.

=> d his

(FILE 'HOME' ENTERED AT 17:28:01 ON 24 MAY 2001)

FILE 'MEDLINE, BIOSIS' ENTERED AT 17:28:10 ON 24 MAY 2001
L1 26 S (FRANCIS J S)/AU
L2 23 DUP REM L1 (3 DUPLICATES REMOVED)
L3 1 S L2 AND CREB
L4 1 S L2 AND CRE

=> d l2 ibib abs 1-23

L2 ANSWER 1 OF 23 MEDLINE
ACCESSION NUMBER: 2000277506 MEDLINE
DOCUMENT NUMBER: 20277506 PubMed ID: 10819355
TITLE: Tungiasis in a young child adopted from South America.
AUTHOR: Darmstadt G L; **Francis J S**
CORPORATE SOURCE: Department of Pediatrics, Children's Hospital and Regional Medical Center, Seattle, WA, USA.
SOURCE: PEDIATRIC INFECTIOUS DISEASE JOURNAL, (2000 May) 19 (5) 485-7.
Journal code: OXJ; 8701858. ISSN: 0891-3668.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200007
ENTRY DATE: Entered STN: 20000728
Last Updated on STN: 20000728
Entered Medline: 20000719

L2 ANSWER 2 OF 23 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 2001:60444 BIOSIS
DOCUMENT NUMBER: PREV200100060444
TITLE: Inhibition of CRE-mediated gene expression by CREB within the context of an apoptotic stimulus.
AUTHOR(S): **Francis, J. S. (1)**; During, M. J.
CORPORATE SOURCE: (1) Thomas Jefferson Univ, Philadelphia, PA USA
SOURCE: Society for Neuroscience Abstracts, (2000) Vol. 26, No. 1-2, pp. Abstract No.-49.16. print.
Meeting Info.: 30th Annual Meeting of the Society of Neuroscience New Orleans, LA, USA November 04-09, 2000 Society for Neuroscience
. ISSN: 0190-5295.
DOCUMENT TYPE: Conference
LANGUAGE: English
SUMMARY LANGUAGE: English
AB Activation of the cAMP response element binding protein (CREB) is a feature of the cellular response to insults that are associated with apoptosis. CREB is thought to activate target gene expression primarily through an interaction with the cAMP response element (CRE) found within the promoter of these genes. An in vitro model of stress-activated cellular signaling was used to investigate the activity of CRE-mediated transcriptional activity within the context of an apoptotic insult. Mouse C17.2 neural precursor cells were transfected with a CRE-containing luciferase reporter cassette prior to exposure to okadiac acid, in order

to investigate [redacted] role of cAMP-mediated transcr[redacted]ional activation within an apoptotic context. Exposure of cells to okadiac acid resulted in a 25-fold induction of luciferase expression that was detectable as early as 15 minutes after exposure, and maximal after 6 hours. Cotransfection of a CRE-CREB cassette significantly reduced okadiac acid- associated induction of luciferase expression. This apparent inhibition by CRE-CREB was reversed by the presence of constitutively expressed dominant-negative CREB mutant (A-CREB), suggesting that this phenomenon is a direct consequence of CREB activity.

L2 ANSWER 3 OF 23 MEDLINE
ACCESSION NUMBER: 1999162195 MEDLINE
DOCUMENT NUMBER: 99162195 PubMed ID: 10053007
TITLE: Cyclic ichthyosis with epidermolytic hyperkeratosis: A phenotype conferred by mutations in the 2B domain of keratin K1.
AUTHOR: Sybert V P; **Francis J S**; Corden L D; Smith L T; Weaver M; Stephens K; McLean W H
CORPORATE SOURCE: Departments of Pediatrics, University of Washington School of Medicine, Children's Hospital and Medical Center, Division of Dermatology, CH-25, 4800 Sand Point Way NE, P.O. Box 5371, Seattle, WA 98105, USA flk01@u.washington.edu Julie S.
CONTRACT NUMBER: AM 62272 (NIADDK)
PO1-AR21557 (NIAMS)
SOURCE: AMERICAN JOURNAL OF HUMAN GENETICS, (1999 Mar) 64 (3) 732-8.
Journal code: 3IM; 0370475. ISSN: 0002-9297.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199904
ENTRY DATE: Entered STN: 19990504
Last Updated on STN: 19990504
Entered Medline: 19990420

AB Bullous congenital ichthyosiform erythroderma (BCIE) is characterized by blistering and erythroderma in infancy and by erythroderma and ichthyosis thereafter. Epidermolytic hyperkeratosis is a hallmark feature of light and electron microscopy. Here we report on four individuals from two families with a unique clinical disorder with histological findings of epidermolytic hyperkeratosis. Manifesting erythema and superficial erosions at birth, which improved during the first few months of life, affected individuals later developed palmoplantar hyperkeratosis with patchy erythema and scale elsewhere on the body. Three affected individuals exhibit dramatic episodic flares of annular, polycyclic erythematous plaques with scale, which coalesce to involve most of the body surface. The flares last weeks to months. In the interim periods the skin may be normal, except for palmoplantar hyperkeratosis. Abnormal keratin-filament aggregates were observed in suprabasal keratinocytes from both probands, suggesting that the causative mutation might reside in keratin K1 or keratin K10. In one proband, sequencing of K1 revealed a heterozygous mutation, 1436T-->C, predicting a change of isoleucine to threonine in the highly conserved helix-termination motif. In the second family, a heterozygous mutation, 1435A-->T, was found in K1, predicting an isoleucine-to-phenylalanine substitution in the same codon. Both mutations were excluded in both a control population and all unaffected family members tested. These findings reveal that a clinical phenotype distinct from classic BCIE but with similar histology can result from K1 mutations and that mutations at this codon give rise to a clinically unique condition.

L2 ANSWER 4 OF 23 BIOSIS COPYRIGHT 2001 BIOSIS

ACCESSION NUMBER: [redacted]7:420000 BIOSIS
DOCUMENT NUMBER: PREV199799719203
TITLE: The HIV-Rev protein utilizes its nuclear export sequence to
interact with nucleoporins Nup49 and Nup100.
AUTHOR(S): **Francis, J. S.**; Scherer, L. J.; Rossi, J. J.
CORPORATE SOURCE: Dep. Molecular Biol., City Hope, Duarte, CA USA
SOURCE: FASEB Journal, (1997) Vol. 11, No. 9, pp. A982.
Meeting Info.: 17th International Congress of Biochemistry and Molecular Biology in conjunction with the Annual Meeting of the American Society for Biochemistry and Molecular Biology San Francisco, California, USA August 24-29, 1997
ISSN: 0892-6638.
DOCUMENT TYPE: Conference; Abstract
LANGUAGE: English

L2 ANSWER 5 OF 23 MEDLINE
ACCESSION NUMBER: 97435953 MEDLINE
DOCUMENT NUMBER: 97435953 PubMed ID: 9290620
TITLE: Spontaneous remission of congenital leukemia.
COMMENT: Comment in: J Pediatr. 1997 Aug;131(2):176-7
AUTHOR: Dinulos J G; Hawkins D S; Clark B S; **Francis J S**
CORPORATE SOURCE: Department of Pediatrics, Children's Hospital and Medical Center, Seattle, WA 98105-0371, USA.
SOURCE: JOURNAL OF PEDIATRICS, (1997 Aug) 131 (2) 300-3. Ref: 17
Journal code: JLZ; 0375410. ISSN: 0022-3476.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW OF REPORTED CASES)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199710
ENTRY DATE: Entered STN: 19971013
Last Updated on STN: 19971013
Entered Medline: 19971002

AB Most patients with congenital leukemia do not survive past infancy despite
aggressive chemotherapy. We describe three patients with congenital leukemia who have undergone prolonged periods of spontaneous remission. Our experience suggests that some patients with congenital leukemia may benefit from initial conservative management without chemotherapy. We summarize the clinical presentations of these patients and review the literature.

L2 ANSWER 6 OF 23 MEDLINE
ACCESSION NUMBER: 97270731 MEDLINE
DOCUMENT NUMBER: 97270731 PubMed ID: 9125766
TITLE: Incontinentia pigmenti.
AUTHOR: **Francis J S**; Sybert V P
CORPORATE SOURCE: Children's Hospital and Medical Center, University of Washington, School of Medicine, Seattle, USA.
SOURCE: SEMINARS IN CUTANEOUS MEDICINE AND SURGERY, (1997 Mar) 16 (1) 54-60. Ref: 59
Journal code: CKV; 9617260. ISSN: 1085-5629.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199706
ENTRY DATE: Entered STN: 19970709
Last Updated on STN: 19970709
Entered Medline: 19970624

AB This article reviews the clinical features, histopathology, genetics, and differential diagnosis of incontinentia pigmenti. Emphasis is placed on appropriate management strategies for patients with incontinentia

pigmenti.

L2 ANSWER 7 OF 23 MEDLINE
ACCESSION NUMBER: 97203049 MEDLINE
DOCUMENT NUMBER: 97203049 PubMed ID: 9050767
TITLE: Indurated purple-red plaque.
AUTHOR: **Francis J S**; Benjamin D R
CORPORATE SOURCE: Department of Medicine, University of Washington, Seattle, USA.
SOURCE: PEDIATRIC DERMATOLOGY, (1997 Jan-Feb) 14 (1) 53-5.
Journal code: PED; 8406799. ISSN: 0736-8046.
PUB. COUNTRY: United States
Conference; (CLINICAL CONFERENCE)
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199705
ENTRY DATE: Entered STN: 19970609
Last Updated on STN: 19970609
Entered Medline: 19970523

L2 ANSWER 8 OF 23 MEDLINE
ACCESSION NUMBER: 96370610 MEDLINE
DOCUMENT NUMBER: 96370610 PubMed ID: 8774506
TITLE: Reiter syndrome initially misdiagnosed as Kawasaki disease.
AUTHOR: Bauman C; Cron R Q; Sherry D D; **Francis J S**
CORPORATE SOURCE: Department of Medicine, University of Washington, Seattle, USA.
SOURCE: JOURNAL OF PEDIATRICS, (1996 Mar) 128 (3) 366-9.
Journal code: JLZ; 0375410. ISSN: 0022-3476.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199701
ENTRY DATE: Entered STN: 19970128
Last Updated on STN: 19970128
Entered Medline: 19970108

AB A misdiagnosis of Kawasaki disease was made initially for two patients with Reiter syndrome. The first patient had conjunctivitis, urethritis, arthritis, and the characteristic skin finding of keratoderma blennorrhagicum. The second patient had conjunctivitis, uveitis, dysuria, arthritis, and the characteristic musculoskeletal finding of enthesitis. Neither patient responded to intravenous immunoglobulin therapy but both responded to nonsteroidal antiinflammatory medication. The clinical characteristics of Reiter syndrome and Kawasaki disease in children are similar but specific features should allow for their differentiation.

L2 ANSWER 9 OF 23 MEDLINE
ACCESSION NUMBER: 96397429 MEDLINE
DOCUMENT NUMBER: 96397429 PubMed ID: 8804323
TITLE: Prevalence of hypopigmented macules in a healthy population.
AUTHOR: Vanderhooft S L; **Francis J S**; Pagon R A; Smith L T; Sybert V P
CORPORATE SOURCE: Department of Medicine (Dermatology), University of Washington School of Medicine, Seattle.
CONTRACT NUMBER: AM 21557 (NIADDK)
AR 07019 (NIAMS)
SOURCE: JOURNAL OF PEDIATRICS, (1996 Sep) 129 (3) 355-61.
Journal code: JLZ; 0375410. ISSN: 0022-3476.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199610
ENTRY DATE: Entered STN: 19961106
Last Updated on STN: 19970203

tered Medline: 19961022

AB OBJECTIVE: Although hypopigmented macules are an important manifestation of tuberous sclerosis (TS), the probability of TS in healthy individuals who have hypopigmented macules is unknown. The purpose of this study was to establish the prevalence of hypopigmented macules among a cross section of the general white population. STUDY DESIGN: The skin of 423 white individuals younger than 45 years of age was screened for hypopigmented macules with ambient incandescent and fluorescent light and a Wood lamp. Indirect ophthalmoscopy was performed in patients with unexplained hypopigmentation to screen for retinal manifestations of TS. RESULTS: Twenty individuals (4.7%) had at least one hypopigmented macule. Of these, four had more than one macule. None had more than three. Two (8%) of the 25 hypopigmented macules were identified only with a Wood lamp. Indirect ophthalmoscopy was performed in 13 (65%) of these 20 individuals. None showed the retinal findings of TS. CONCLUSIONS: The prevalence of hypopigmented macules in the general population has been underestimated. The presence of a few hypopigmented macules on the skin of an otherwise healthy individual without a family history of TS need not prompt an evaluation to rule out this disorder.

L2 ANSWER 10 OF 23 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1996:455189 BIOSIS
DOCUMENT NUMBER: PREV199699177545
TITLE: Use of cDNA selection to develop a transcription map of the haemochromatosis gene region.
AUTHOR(S): Goldwurm, S. (1); Smit, D. J.; **Francis, J. S.**; Burt, M. J.; Powell, L. W.; Jazwinska, E. C.
CORPORATE SOURCE: (1) Liver Unit, Queensland Inst. Med. Res., Brisbane, QLD Australia
SOURCE: Italian Journal of Gastroenterology, (1996) Vol. 28, No. 5, pp. 296.
Meeting Info.: The Italian Association for the Study of the Liver, Spring Meeting Rome, Italy July 3-5, 1996
ISSN: 0392-0623.
DOCUMENT TYPE: Conference
LANGUAGE: English

L2 ANSWER 11 OF 23 MEDLINE
ACCESSION NUMBER: 96319535 MEDLINE
DOCUMENT NUMBER: 96319535 PubMed ID: 8708037
TITLE: Cimetidine therapy for multiple viral warts in children.
COMMENT: Comment in: J Am Acad Dermatol. 1997 Aug;37(2 Pt 1):289-90
AUTHOR: Bauman C; **Francis J S**; Vanderhooft S; Sybert V P
CORPORATE SOURCE: Department of Medicine, University of Washington School of Medicine, Seattle, USA.
SOURCE: JOURNAL OF THE AMERICAN ACADEMY OF DERMATOLOGY, (1996 Aug) 35 (2 Pt 1) 271-2.
Journal code: HVG; 7907132. ISSN: 0190-9622.
PUB. COUNTRY: United States
(CLINICAL TRIAL)
Journal; Article; (JOURNAL ARTICLE)
(RANDOMIZED CONTROLLED TRIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199609
ENTRY DATE: Entered STN: 19960919
Last Updated on STN: 19980206
Entered Medline: 19960910

L2 ANSWER 12 OF 23 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1996:555168 BIOSIS
DOCUMENT NUMBER: PREV199699277524
TITLE: Two novel mutations in K1 codon 479 cause a unique form of ichthyosis.

AUTHOR(S): [redacted]ncis, J. S. (1); Smith, L. T. [redacted]; Sybert, V. P. (1); Stephens, K.; Corden, L. D.; McLean, W. H. I.
CORPORATE SOURCE: (1) Univ. Washington, Seattle, WA USA
SOURCE: American Journal of Human Genetics, (1996) Vol. 59, No. 4 SUPPL., pp. A38.
Meeting Info.: 46th Annual Meeting of the American Society of Human Genetics San Francisco, California, USA October 29-November 2, 1996
ISSN: 0002-9297.
DOCUMENT TYPE: Conference
LANGUAGE: English

L2 ANSWER 13 OF 23 MEDLINE
ACCESSION NUMBER: 95243671 MEDLINE
DOCUMENT NUMBER: 95243671 PubMed ID: 7726588
TITLE: Familial pityriasis rubra pilaris.
AUTHOR: Vanderhooft S L; **Francis J S**; Holbrook K A; Dale B A; Fleckman P
CORPORATE SOURCE: Department of Medicine, University of Washington School of Medicine, Seattle, USA.
CONTRACT NUMBER: 5 T32 AR07019-19 (NIAMS)
AR-21557 (NIAMS)
SOURCE: ARCHIVES OF DERMATOLOGY, (1995 Apr) 131 (4) 448-53.
Journal code: 6WU; 0372433. ISSN: 0003-987X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199505
ENTRY DATE: Entered STN: 19950605
Last Updated on STN: 19950605
Entered Medline: 19950519

AB BACKGROUND: Familial pityriasis rubra pilaris is a rare autosomal dominant skin disorder. Four individuals from one family are described who demonstrate clinical features compatible with a diagnosis of familial pityriasis rubra pilaris. Results of light and electron microscopic, immunocytochemical, and biochemical analysis of skin biopsy specimens from three of these four individuals are presented. OBSERVATIONS: All affected individuals demonstrated erythematous scaly skin with follicular prominence and islands of sparing. Inheritance was consistent with an autosomal dominant trait. Light and electron microscopic findings were compatible with those reported in sporadic cases of pityriasis rubra pilaris. Immunocytochemistry showed suprabasal staining with monoclonal antibody AE1. Immunoblot analysis revealed abnormal keratins with K6/16 expression, the possibility of an abnormal K14 or K16, and a 45-kd acidic keratin not normally expressed in epidermis. Because similar biochemical analyses have not been reported previously in other cases of pityriasis rubra pilaris (familial or sporadic), comparisons cannot be made. CONCLUSIONS: The observations suggest that the cutaneous abnormality in this family extends beyond clinical and morphological alterations to abnormalities in biochemical markers of epidermal differentiation.

L2 ANSWER 14 OF 23 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 95397819 MEDLINE
DOCUMENT NUMBER: 95397819 PubMed ID: 7668262
TITLE: Evidence that the ancestral haplotype in Australian hemochromatosis patients may be associated with a common mutation in the gene.
AUTHOR: Crawford D H; Powell L W; Leggett B A; **Francis J S**; Fletcher L M; Webb S I; Halliday J W; Jazwinska E C
CORPORATE SOURCE: Joint Liver Program, Queensland Institute of Medical Research, Brisbane, Australia.
SOURCE: AMERICAN JOURNAL OF HUMAN GENETICS, (1995 Aug) 57 (2) 362-7.
Journal code: 3IM; 0370475. ISSN: 0002-9297.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199510
ENTRY DATE: Entered STN: 19951020
Last Updated on STN: 19970203
Entered Medline: 19951012

AB Hemochromatosis (HC) is a common inherited disorder of iron metabolism for which neither the gene nor biochemical defect have yet been identified. The aim of this study was to look for clinical evidence that the predominant ancestral haplotype in Australian patients is associated with a common mutation in the gene. We compared indices of iron metabolism and storage in three groups of HC patients categorized according to the presence of the ancestral haplotype (i.e., patients with two copies, one copy, and no copies of the ancestral haplotype). We also examined iron indices in two groups of HC heterozygotes (those with the ancestral haplotype and those without) and in age-matched controls. These analyses indicate that (i) HC patients with two copies of the ancestral haplotype show significantly more severe expression of the disorder than those with one copy or those without, (ii) HC heterozygotes have partial clinical expression, which may be influenced by the presence of the ancestral haplotype in females but not in males, and (iii) the high population frequency of the HC gene may be the result of the selective advantage conferred by protecting heterozygotes against iron deficiency.

L2 ANSWER 15 OF 23 MEDLINE
ACCESSION NUMBER: 95039253 MEDLINE
DOCUMENT NUMBER: 95039253 PubMed ID: 7951667
TITLE: Genetic skin diseases.
AUTHOR: **Francis J S**
CORPORATE SOURCE: University of Washington, Seattle.
SOURCE: CURRENT OPINION IN PEDIATRICS, (1994 Aug) 6 (4) 447-53.
Ref: 63
Journal code: BUT; 9000850. ISSN: 1040-8703.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199412
ENTRY DATE: Entered STN: 19950110
Last Updated on STN: 19950110
Entered Medline: 19941202

AB Recent advances in molecular genetics have led to major breakthroughs in the understanding of two heterogeneous groups of inherited skin diseases, epidermolysis bullosa and the ichthyoses. Mutations in keratins K5 or K14 are found in epidermolysis bullosa simplex. The gravis (Herlitz) variety of junctional epidermolysis bullosa is characterized by defects in the anchoring filament protein kalinin. Both dominant and recessive forms of dystrophic epidermolysis bullosa appear to be due to mutations in the type VII collagen gene. Biochemical studies in patients with ichthyosis vulgaris reveal that the proteins profilaggrin and filaggrin are reduced or absent. Recessive X-linked ichthyosis is characterized by a deficiency of the enzyme steroid sulfatase. A type of lamellar ichthyosis may be explained on the basis of abnormal cornified cell envelope formation, and bullous congenital ichthyosiform erythroderma (epidermolytic hyperkeratosis) is caused by mutations in keratins K1 or K10.

L2 ANSWER 16 OF 23 MEDLINE
ACCESSION NUMBER: 94134598 MEDLINE
DOCUMENT NUMBER: 94134598 PubMed ID: 8302749
TITLE: Scrotal hair growth in infancy.
COMMENT: Comment on: Pediatr Dermatol. 1993 Mar;10(1):34-5
AUTHOR: **Francis J S**; Ruvalcaba R H
SOURCE: PEDIATRIC DERMATOLOGY, (1993 Dec) 10 (4) 389-90.
Journal code: PED; 8406799. ISSN: 0736-8046.
PUB. COUNTRY: United States

mentary
Letter
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199403
ENTRY DATE: Entered STN: 19940318
Last Updated on STN: 19950206
Entered Medline: 19940307

L2 ANSWER 17 OF 23 MEDLINE
ACCESSION NUMBER: 90138520 MEDLINE
DOCUMENT NUMBER: 90138520 PubMed ID: 2694129
TITLE: Congenital monocytic leukemia: report of a case with cutaneous involvement, and review of the literature.
AUTHOR: **Francis J S**; Sybert V P; Benjamin D R
CORPORATE SOURCE: Department of Medicine, University of Washington, School of
Medicine, Seattle 98195.
SOURCE: PEDIATRIC DERMATOLOGY, (1989 Dec) 6 (4) 306-11. Ref: 25
Journal code: PED; 8406799. ISSN: 0736-8046.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW OF REPORTED CASES)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199003
ENTRY DATE: Entered STN: 19900328
Last Updated on STN: 19900328
Entered Medline: 19900309
AB Congenital leukemia is a rare disease that can become manifest soon after birth. Cutaneous involvement consists of red, brown, or purple papules and
nodules, and confluent areas of purpura. The diagnosis is established by the presence of leukemic cells in biopsy specimens of bone marrow and involved skin, and by immunocytochemical characterization of these cells. We report a case of congenital monocytic leukemia with a normal karyotype,
whose disease underwent temporary spontaneous regression.

L2 ANSWER 18 OF 23 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 82026769 MEDLINE
DOCUMENT NUMBER: 82026769 PubMed ID: 7285720
TITLE: Cardiovascular effects of chronic intraventricular administration of angiotensin II in dogs.
AUTHOR: Buckley J P; Lokhandwala M F; Steenberg M; **Francis J S**; Tadepalli A S
SOURCE: CLINICAL AND EXPERIMENTAL HYPERTENSION, (1981) 3 (5) 1001-18.
Journal code: D9V; 7803060. ISSN: 0148-3927.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198112
ENTRY DATE: Entered STN: 19900316
Last Updated on STN: 19900316
Entered Medline: 19811215
AB Intracerebroventricular administration of angiotensin II (AII), 1 microgram twice a day to mongrel dogs plus saline as the drinking fluid for 4 weeks produced a significant sustained elevation in systolic and diastolic blood pressures. The hypertensive state appeared to be due to an
increase in total peripheral resistance. Fluid intake and urine output were elevated and there was a significant increase in body weight at the end of week 2, 3 and 4. Serum Na+ levels were significantly decreased and serum Ca++ levels significantly increased in the hypertensive animals. These studies indicate that increasing AII levels in the cerebrospinal fluid for a prolonged period of time produces a sustained hypertensive

state only if t[redacted]daily intake of sodium is incr[redacted]ed and that the alterations in vascular resistance may be due to changes in the Na+ - Ca++ fluxes.

L2 ANSWER 19 OF 23 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1983:66595 BIOSIS
DOCUMENT NUMBER: BR24:66595
TITLE: CIRCULATORY EFFECTS OF CHRONIC INTRA VENTRICULAR ADMINISTRATION OF ANGIOTENSIN II IN DOGS.
AUTHOR(S): BUCKLEY J P; LOKHANDWALA M F; JANDHYALA B S; **FRANCIS J S**; TADEPALLI A
CORPORATE SOURCE: DEP. PHARMACOL., INST. CARDIOVASC. STUDIES, UNIV. HOUSTON, HOUSTON, TEX. 77004.
SOURCE: BUCKLEY, J. P. AND C. M. FERRARIO (ED.). PERSPECTIVES IN CARDIOVASCULAR RESEARCH, VOL. 6. CENTRAL NERVOUS SYSTEM MECHANISMS IN HYPERTENSION; MEETING, HOUSTON, TEX., USA, MAY 1980. XVIII+416P. RAVEN PRESS: NEW YORK, N.Y., USA, (1981) 0 (0), P363-376.
CODEN: PCRED9. ISSN: 0361-0527. ISBN: 0-89004-545-3.
FILE SEGMENT: BR; OLD
LANGUAGE: English

L2 ANSWER 20 OF 23 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1978:107036 BIOSIS
DOCUMENT NUMBER: BR15:50536
TITLE: BLOCKADE BY SARCOSINE 1 ALANINE 8 ANGIOTENSIN II OF CENTRAL
ANGIOTENSIN II PRODUCED CARDIO VASCULAR EFFECTS.
AUTHOR(S): **FRANCIS J S**; TADEPALLI A S; BUCKLEY J P
SOURCE: Pharmacologist, (1978) 20 (3), 207.
CODEN: PHMCAA. ISSN: 0031-7004.
DOCUMENT TYPE: Conference
FILE SEGMENT: BR; OLD
LANGUAGE: Unavailable

L2 ANSWER 21 OF 23 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1978:132577 BIOSIS
DOCUMENT NUMBER: BA65:19577
TITLE: DIFFERENTIAL CARDIO VASCULAR CHANGES AS A FUNCTION OF STIMULATION ELECTRODE SITE IN RABBIT HYPOTHALAMUS.
AUTHOR(S): SAMPSON L D; SCHNEIDERMAN N; WALLACH J; GAVIN W J; **FRANCIS J S**
CORPORATE SOURCE: DEP. PSYCHOL., UNIV. MIAMI, CORAL GABLES, FLA. 33124, USA.
SOURCE: PHYSIOL BEHAV, (1977) 19 (1), 111-120.
CODEN: PHBHA4. ISSN: 0031-9384.
FILE SEGMENT: BA; OLD
LANGUAGE: English
AB Chronically prepared, unanesthetized rabbits (20) received high-frequency (200 pulse/s), short pulse-train (1.0 s) and relatively low-frequency (25 pulse-s), long pulse-train (10 s) electrical stimulation of the hypothalamus. High-frequency, short pulse train stimulation elicited a pressor response and bradycardia at all 27 electrode sites. Three other cardiovascular response patterns were obtained following low-frequency, long pulse-train stimulation. These latter patterns reflected a medial-lateral organization of autonomic function within the hypothalamus.
Whereas all 15 lateral hypothalamic placements yielded depressor responses, 7 of 12 medial hypothalamic placements yielded pressor responses and tachycardia. Cardiovascular changes following administration
of selective autonomic blocking agents (e.g., phentolamine, propranolol, methylatropine) suggest that high-frequency, short pulse-train stimulation
elicited a pressor response followed by a reflexive bradycardia essentially mediated by an increase in vagal restraint. The heart rate changes observed to low-frequency, long pulse-train stimulation appear to have been importantly influenced by changes at the heart in .beta.-adrenergic activity.

L2 ANSWER 22 OF 23 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 73248539 MEDLINE
DOCUMENT NUMBER: 73248539 PubMed ID: 4580801
TITLE: Cardiovascular responses of rabbits to ESB: effects of anesthetization, stimulus frequency and pulse-train duration.
AUTHOR: **Francis J S**; Sampson L D; Gerace T Sr; Schneiderman N
SOURCE: PHYSIOLOGY AND BEHAVIOR, (1973 Aug) 11 (2) 195-203. Journal code: P72; 0151504. ISSN: 0031-9384.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 197311
ENTRY DATE: Entered STN: 19900310
Last Updated on STN: 19900310
Entered Medline: 19731106

L2 ANSWER 23 OF 23 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1969:17296 BIOSIS
DOCUMENT NUMBER: BR05:17296
TITLE: COMPOSTING A BRIDGE BETWEEN BIO-DYNAMICS AND ORTHODOX METHODS SOIL.
AUTHOR(S): **FRANCIS J S**
SOURCE: Bio-Dynamics, (1967) (84), 18-22.
CODEN: BIDSA2. ISSN: 0006-2863.
FILE SEGMENT: BR; OLD
LANGUAGE: Unavailable

=> s (during m j)/au

L5 163 (DURING M J)/AU

=> s l5 and cre?

L6 6 L5 AND CRE?

=> dup rem l6

PROCESSING COMPLETED FOR L6
L7 5 DUP REM L6 (1 DUPLICATE REMOVED)

=> d ibib abs 1-5

L7 ANSWER 1 OF 5 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 2001:109506 BIOSIS
DOCUMENT NUMBER: PREV200100109506
TITLE: Direct gene transfer of **CREB** promotes survival of nigrostriatal neurons in a rat model of Parkinson disease.
AUTHOR(S): Mouravlev, A. (1); Dunning, J.; Zarkovic, A.; Mastakov, M.; Xu, R. A.; Dragunow, M.; **During, M. J.**
CORPORATE SOURCE: (1) University of Auckland, Auckland New Zealand
SOURCE: Society for Neuroscience Abstracts, (2000) Vol. 26, No. 1-2, pp. Abstract No.-700.5. print.
Meeting Info.: 30th Annual Meeting of the Society of Neuroscience New Orleans, LA, USA November 04-09, 2000 Society for Neuroscience
. ISSN: 0190-5295.
DOCUMENT TYPE: Conference
LANGUAGE: English
SUMMARY LANGUAGE: English
AB The cyclic AMP-responsive element binding protein (**CREB**) is a transcription factor induced by extracellular stimuli and is implicated in

diverse brain f[redacted]tions including cell survival. [redacted]eviously we showed that
transient **CREB** overexpression altered the susceptibility of neurons to apoptosis. We found that elevated **CREB** protein inhibited apoptosis induced by okadaic acid in vitro (M. Walton et al., 1999). In this study we investigated whether the in vivo gene transfer of **CREB** into substantia nigra pars compacta (SNc) preceding medial forebrain bundle (MFB) lesion with 6-OHDA promoted survival of dopaminergic nigrostriatal neurons. We used recombinant AAV vectors with expression cassettes carrying a **CREB** cDNA as well as its dominant negative mutants mCREB (with substitution of Ala to Ser133) and A-**CREB** (S. Ahn et al., 1998). Both a GFP-expressing vector and sham injections were used as controls. Microinjection of the **CREB** vector into rat SNc significantly reduced asymmetrical drug-induced rotation (by 37%) and paw preference behavior (by 53%) caused by the lesion. The survival of dopaminergic neurons in SNc and the tyrosine hydroxylase immuno-reactivity in the ipsilateral striatum was significantly higher in animals injected with **CREB** vector compared with control groups. These results suggest that **CREB** overexpression in the SNc protects dopaminergic nigrostriatal neurons against toxin-induced apoptosis.

L7 ANSWER 2 OF 5 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 2001:60444 BIOSIS
DOCUMENT NUMBER: PREV200100060444
TITLE: Inhibition of **CRE**-mediated gene expression by **CREB** within the context of an apoptotic stimulus.
AUTHOR(S): Francis, J. S. (1); **During, M. J.**
CORPORATE SOURCE: (1) Thomas Jefferson Univ, Philadelphia, PA USA
SOURCE: Society for Neuroscience Abstracts, (2000) Vol. 26, No. 1-2, pp. Abstract No.-49.16. print.
Meeting Info.: 30th Annual Meeting of the Society of Neuroscience New Orleans, LA, USA November 04-09, 2000 Society for Neuroscience
. ISSN: 0190-5295.
DOCUMENT TYPE: Conference
LANGUAGE: English
SUMMARY LANGUAGE: English
AB Activation of the cAMP response element binding protein (**CREB**) is a feature of the cellular response to insults that are associated with apoptosis. **CREB** is thought to activate target gene expression primarily through an interaction with the cAMP response element (**CRE**) found within the promoter of these genes. An in vitro model of stress-activated cellular signaling was used to investigate the activity of **CRE**-mediated transcriptional activity within the context of an apoptotic insult. Mouse C17.2 neural precursor cells were transfected with a **CRE**-containing luciferase reporter cassette prior to exposure to okadiac acid, in order to investigate the role of cAMP-mediated transcriptional activation within an apoptotic context. Exposure of cells to okadiac acid resulted in a 25-fold induction of luciferase expression that was detectable as early as 15 minutes after exposure, and maximal after 6 hours. Cotransfection of a **CRE-CREB** cassette significantly reduced okadiac acid- associated induction of luciferase expression. This apparent inhibition by **CRE-CREB** was reversed by the presence of constitutively expressed dominant-negative **CREB** mutant (A-**CREB**), suggesting that this phenomenon is a direct consequence of **CREB** activity.

L7 ANSWER 3 OF 5 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 2001:80913 BIOSIS
DOCUMENT NUMBER: PREV200100080913
TITLE: Gilatide: a novel peptide with memory enhancing properties.
AUTHOR(S): Haile, C. N. (1); **During, M. J.**
CORPORATE SOURCE: (1) Thomas Jefferson Medical College, Philadelphia, PA USA
SOURCE: Society for Neuroscience Abstracts, (2000) Vol. 26, No. 1-2, pp. Abstract No.-373.4. print.
Meeting Info.: 30th Annual Meeting of the Society of

[redacted]roscience New Orleans, LA, USA [redacted]vember 04-09, 2000
Society for Neuroscience
. ISSN: 0190-5295.
DOCUMENT TYPE: Conference
LANGUAGE: English
SUMMARY LANGUAGE: English
AB Several neuropeptides facilitate learning and memory, but most have characteristics that preclude clinical use. To address this, we designed a truncated peptide (Gilatide, GILA) consisting of 9 amino acid residues homologous with discrete domains of peptides (exendin-4, glucagon-like peptide) known to stimulate the cAMP-**CREB** cascade; a pathway involved in learning and memory. We determined whether GILA enhances associative learning using a single-trial passive avoidance paradigm. Male Sprague Dawley rats were anesthetized with isofluorane then administered GILA (0.1-60ug, in 5% methyl-beta-cylodextrin, N=5-13) or vehicle (VEH) intra-nasally, or injected with nicotine (0.3mg/kg, sc, NIC). Rats were then placed in the light side of a 2-chambered light/dark Plexiglas apparatus. Once the rat entered the dark chamber, a guillotine door was closed and a 1.0mA shock (3sec) was administered. The door was then raised and the rat was allowed to re-enter the light side. Latency (max 600sec) to enter the dark chamber was measured at 1, 3, 7 and 21 days post pairing. Compared to VEH (276+-58), GILA (10ug, 542+-52) and NIC (445+-84) produced greater latency times at 1 day (P<0.05). At 3 days, GILA (10ug) produced greater (458+77,P<0.05) latency times compared to NIC (219+-115) and vehicle (136+-41). At 7 days, GILA (10ug) continued to show greater latency (501+-47,P<0.05) compared to NIC (263+-75) and VEH (210+-64). There were no differences between groups at 21 days. Co-treatment with the exendin-4 (9-39) antagonist (10ug) blocked the memory enhancing effects of GILA (P<0.05, 216+-89) when tested at 1 day. Increasing doses of GILA (20ug) surmounted the antagonism of the exendin -4 antagonist (550+49,P<0.01). GILA (10ug) had no effects on nociception or locomotor activity. Thus, intra-nasally administered GILA exhibits potent associative memory enhancing effects and this appears to be mediated through an exendin-4 receptor mechanism.

L7 ANSWER 4 OF 5 MEDLINE
ACCESSION NUMBER: 2000005433 MEDLINE
DOCUMENT NUMBER: 20005433 PubMed ID: 10537041
TITLE: **CREB** phosphorylation promotes nerve cell survival.
AUTHOR: Walton M; Woodgate A M; Muravlev A; Xu R; **During M J**; Dragunow M
CORPORATE SOURCE: Department of Pharmacology, Faculty of Medicine and Health Science, University of Auckland, New Zealand.
SOURCE: JOURNAL OF NEUROCHEMISTRY, (1999 Nov) 73 (5) 1836-42.
Journal code: JAV; 2985190R. ISSN: 0022-3042.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199911
ENTRY DATE: Entered STN: 20000111
Last Updated on STN: 20000111
Entered Medline: 19991105
AB The cyclic AMP-responsive element binding protein (**CREB**) is a posttranslationally activated transcription factor that has been implicated in numerous brain functions including cell survival. In this study we investigated whether **CREB** overexpression using transient transfection of a pAAV/CMV-**CREB** plasmid altered neuronal cells' susceptibility to apoptosis. We found that elevated **CREB** protein inhibited apoptosis induced by okadaic acid. At least part of this effect is critically dependent on prolonged Ser133 phosphorylation, as a directed mutation at this site decreased

CREB-induced pr[redacted]ction. These results suggest t[redacted] **CREB** is a survival factor for neuronal cells and that treatments aimed at augmenting **CREB** phosphorylation in the brain may be neuroprotective.

L7 ANSWER 5 OF 5 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 89153352 MEDLINE
DOCUMENT NUMBER: 89153352 PubMed ID: 2920789
TITLE: Controlled release of dopamine from a polymeric brain implant: in vitro characterization.
AUTHOR: Freese A; Sabel B A; Saltzman W M; **During M J**; Langer R
CORPORATE SOURCE: Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge 02139.
CONTRACT NUMBER: GM26698 (NIGMS)
SOURCE: EXPERIMENTAL NEUROLOGY, (1989 Mar) 103 (3) 234-8. Journal code: EQF; 0370712. ISSN: 0014-4886.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198904
ENTRY DATE: Entered STN: 19900306
Last Updated on STN: 19980206
Entered Medline: 19890413

AB A biocompatible polymeric matrix system for the long-term controlled release of dopamine has been developed. Solid particles of this bioactive agent were encapsulated in ethylene-vinyl acetate copolymer (EVAc). Following immersion in an aqueous buffer solution, the release rate of dopamine from the polymer matrix was found to depend on the initial concentration of dopamine in the polymer. After coating the matrix devices with an additional impermeable layer of EVAc, constant rates of release were obtained by **creating** a cavity in this impermeable layer. The observed experiments are consistent with a diffusion-limited model of dopamine release; all the in vitro experimental results were therefore correlated by the effective diffusion coefficient of dopamine through the porous polymer network. These results are discussed in terms of potential design modifications to achieve desired release characteristics for a variety of neuroactive substances, including neurotransmitters or their precursors.

=> s (walton, M)/au

L8 166 (WALTON, M)/AU

=> s l8 and creb

L9 6 L8 AND CREB

=> dup rem l9

PROCESSING COMPLETED FOR L9
L10 4 DUP REM L9 (2 DUPLICATES REMOVED)

=> d ibib abs 1-4

L10 ANSWER 1 OF 4 MEDLINE
ACCESSION NUMBER: 2000005433 MEDLINE
DOCUMENT NUMBER: 20005433 PubMed ID: 10537041
TITLE: **CREB** phosphorylation promotes nerve cell survival.
AUTHOR: **Walton M**; Woodgate A M; Muravlev A; Xu R; During M J; Dragunow M
CORPORATE SOURCE: Department of Pharmacology, Faculty of Medicine and Health Science, University of Auckland, New Zealand.
SOURCE: JOURNAL OF NEUROCHEMISTRY, (1999 Nov) 73 (5) 1836-42.

rnal code: JAV; 2985190R. ISSN 022-3042.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199911
ENTRY DATE: Entered STN: 20000111
Last Updated on STN: 20000111
Entered Medline: 19991105

AB The cyclic AMP-responsive element binding protein (**CREB**) is a posttranslationally activated transcription factor that has been implicated in numerous brain functions including cell survival. In this study we investigated whether **CREB** overexpression using transient transfection of a pAAV/CMV-**CREB** plasmid altered neuronal cells' susceptibility to apoptosis. We found that elevated **CREB** protein inhibited apoptosis induced by okadaic acid. At least part of this effect is critically dependent on prolonged Ser133 phosphorylation, as a directed mutation at this site decreased **CREB**-induced protection. These results suggest that **CREB** is a survival factor for neuronal cells and that treatments aimed at augmenting **CREB** phosphorylation in the brain may be neuroprotective.

L10 ANSWER 2 OF 4 MEDLINE
ACCESSION NUMBER: 1999227185 MEDLINE
DOCUMENT NUMBER: 99227185 PubMed ID: 10209230
TITLE: Neuronal death and survival in two models of hypoxic-ischemic brain damage.
AUTHOR: **Walton M**; Connor B; Lawlor P; Young D; Sirimanne E; Gluckman P; Cole G; Dragunow M
CORPORATE SOURCE: Department of Pharmacology, Faculty of Medicine and Health Science, University of Auckland, Auckland, New Zealand.
SOURCE: BRAIN RESEARCH. BRAIN RESEARCH REVIEWS, (1999 Apr) 29 (2-3)
137-68. Ref: 388
Journal code: BRS; 8908638. ISSN: 0165-0173.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, ACADEMIC)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199906
ENTRY DATE: Entered STN: 19990614
Last Updated on STN: 20000303
Entered Medline: 19990603

AB Two unilateral hypoxic-ischemia (HI) models (moderate and severe) in immature rat brain have been used to investigate the role of various transcription factors and related proteins in delayed neuronal death and survival. The moderate HI model results in an apoptotic-like neuronal death in selectively vulnerable regions of the brain while the more severe HI injury consistently produces widespread necrosis resulting in infarction, with some necrosis resistant cell populations showing evidence of an apoptotic type death. In susceptible regions undergoing an apoptotic-like death there was not only a prolonged induction of the immediate early genes, c-jun, c-fos and nur77, but also of possible target genes amyloid precursor protein (APP751) and CPP32. In contrast, increased levels of BDNF, phosphorylated **CREB** and PGHS-2 were found in cells resistant to the moderate HI insult suggesting that these proteins either alone or in combination may be of importance in the process of neuroprotection. An additional feature of both the moderate and severe brain insults was the rapid activation and/or proliferation of glial cells (microglia and astrocytes) in and around the site of damage. The glial response following HI was associated with an upregulation of both the

CCAAT-enhancer [redacted]ding protein alpha (microglia [redacted]y) and NFkappaB transcription factors.
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L10 ANSWER 3 OF 4 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 1999423712 MEDLINE
DOCUMENT NUMBER: 99423712 PubMed ID: 10491575
TITLE: Immediate early gene transcription and synaptic modulation.
AUTHOR: **Walton M**; Henderson C; Mason-Parker S; Lawlor P; Abraham W C; Bilkey D; Dragunow M
CORPORATE SOURCE: Department of Pharmacology, Faculty of Medicine and Health Science, University of Auckland, Auckland, New Zealand.
SOURCE: JOURNAL OF NEUROSCIENCE RESEARCH, (1999 Oct 1) 58 (1) 96-106. Ref: 99
Journal code: KAC; 7600111. ISSN: 0360-4012.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
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LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199911
ENTRY DATE: Entered STN: 20000111
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Entered Medline: 19991104

AB Long-term changes in gene expression appear to be critical to the formation of memory, but little is known about its stimulus-transcription coupling. Numerous studies in the last decade, by focusing on unraveling this signal transduction pathway, have investigated the potential role of the immediate-early genes in this process. The krox family of immediate-early gene proteins are of particular interest because they may be involved in stabilizing the synaptic modifications that underlie hippocampal long-term potentiation (LTP). A potential upstream mediator of krox induction is cyclic AMP-responsive element binding protein (**CREB**), a posttranslationally activated transcription factor that has been implicated in numerous memory paradigms. In this study we investigated whether the activation of **CREB** by phosphorylation may have a role in the development of rat perforant- path-stimulated LTP and associated dentate granule cell krox-24 mRNA expression. Contrary to what was expected, we failed to show any difference in the levels of phosphorylated **CREB** after LTP or following endogenous synaptic facilitation stimulated by novelty. Using these same model systems we also investigated the protein levels of brain- derived neurotrophic factor (BDNF), another immediate-early gene that is induced following a durable form of LTP. However, BDNF protein was not induced within the hippocampus after LTP and was transiently decreased following novel environmental stimulation.

L10 ANSWER 4 OF 4 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 97189244 MEDLINE
DOCUMENT NUMBER: 97189244 PubMed ID: 9037515
TITLE: The role of the cyclic AMP-responsive element binding protein (**CREB**) in hypoxic-ischemic brain damage and repair.
AUTHOR: **Walton M**; Sirimanne E; Williams C; Gluckman P; Dragunow M
CORPORATE SOURCE: Department of Pharmacology and Clinical Pharmacology, Faculty of Medicine and Health Science, University of Auckland, New Zealand.
SOURCE: BRAIN RESEARCH. MOLECULAR BRAIN RESEARCH, (1996 Dec 31) 43 (1-2) 21-9.
Journal code: MBR; 8908640. ISSN: 0169-328X.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199705
ENTRY DATE: Entered STN: 19970609
Last Updated on STN: 20000303
Entered Medline: 19970529

AB The cyclic AMP-responsive element binding protein (**CREB**) is a basally expressed, post-translationally activated transcription factor that has been implicated in the trans-activation of a number of genes in response to cAMP and calcium signals. A unilateral hypoxic-ischemic (HI) injury in the 21 day old rat was used to examine a potential role for **CREB** (phosphorylated and unphosphorylated) in neuronal programmed cell death or cell survival. The selectively vulnerable CAI pyramidal cells, which undergo delayed neuronal death following mild HI, show a loss of **CREB** and phosphorylated **CREB** (pCREB) immunoreactivity on the injured side 48 and 72 h following HI. In contrast the resistant dentate granule cells and cortical cells produce a bimodal increase in pCREB immunoreactivity, peaking 6 and 48 h following HI. The fact that cells surviving the HI insult are showing increased activation of **CREB** suggests that this protein might be involved in the process of neuroprotection.

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Vallejo, et al., Molecular and cellular Biology 15(1)415-424, 1995

Kim KS et al., J. Neuroscience 14(11 pt 2) 7200-7, 1994

Walton M ; Woodgate A M; Muravlev A; Xu R; During M J; Dragunow M
JOURNAL OF NEUROCHEMISTRY, (1999 Nov) 73 (5) 1836-42.
Journal code: J

Dobbeling u et al., FEBS Lett. 391(1-2)131-3, 1996

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Thank you

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Proliferation of Hepatic Stellate Cells Is Inhibited by Phosphorylation of CREB on Serine 133

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Department of Medicine, Veterans Affairs Medical Center, and Center for Molecular Genetics, University of California, San Diego, California 92161

Abstract

Proliferating, activated, hepatic stellate cells have a high level of collagen type I expression. Therefore, stellate cell proliferation is a critical step in hepatic fibrosis. Here we show that proliferation of activated primary rat stellate cells was blocked by elevation of cAMP with 8 Br-cAMP or isomethylbutyl xanthine, a phosphodiesterase inhibitor, and by stimulation of Ca^{2+} fluxes with the Ca^{2+} ionophore A-23187. Because phosphorylation of CREB on Ser133 is an important mediator of cAMP-protein kinase (PKA) and Ca^{2+}-calmodulin kinase II (CAMK-II) activation, we tested whether CREB-PSer133 was essential for stellate cell quiescence. Nuclear extracts from quiescent, but not from activated, stellate cells contained CREB-PSer133. Moreover, the phosphorylation of CREB on Ser133 was stimulated in activated cells by inducing the activity of PKA or CAMK-II. In addition, coexpression of CREB and either a constitutively active PKA or a constitutively active CAMK-II inhibited the proliferation of activated stellate cells. In contrast, expression of CREB alone, PKA or CAMK-II alone, CREB-Ala 133 (which lacks the Ser133 phosphoacceptor) with PKA or CAMK-II, or CREB with inactive PKA or CAMK-II mutants did not affect stellate cell proliferation, suggesting that CREB-PSer133 is necessary for blocking the stellate cell cycle. Conversely, expression of a *trans*-dominant negative CREB-Ala 133 mutant (which competes with CREB/CREB-PSer133 for cognate DNA binding sites and presumably for protein interactions) induced a greater than fivefold entry into S-phase of quiescent stellate cells, compared with control cells expressing either β-galactosidase or wt CREB, indicating that CREB-PSer133 may be indispensable for the quiescent stellate cell phenotype. This study suggests that PKA and CAMK-II play an essential role on stellate cell activation through the induction of CREB phosphorylation on Ser133, and provides potential approaches for the treatment of hepatic fibrogenesis in patients with chronic liver diseases. (*J. Clin. Invest.* 1997. 99:1322–1328.) Key words: hepatic stellate cell proliferation • liver fibrogenesis • CREB phosphorylation • protein kinase A • calcium calmodulin kinase-II

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Introduction

Collagen type I is excessively deposited in the extracellular matrix protein in hepatic fibrosis (1, 2), which in turn contributes to the morbidity and mortality of patients with chronic liver diseases (3). Hepatic stellate cells play a key role in the pathogenesis of hepatic fibrosis (4, 5). Although we (6) and others (7, 8) reported that quiescent stellate cells produce little collagen type I, proliferating, activated (myofibroblastic) stellate cells display a high level of collagen α_1(I) gene expression. In addition, the degree of fibrogenesis in liver diseases is most likely affected by the increased population of stellate cells, which results from their proliferation (9).

Therefore, stellate cell proliferation is a critical step in hepatic fibrogenesis. Studies with primary cultures of adult rat stellate cells have provided evidence that cell type–specific mechanisms modulate their proliferation (10–12). For example, the induction of stellate cell proliferation by TGFα or collagen type I matrix is mediated by oxidative stress through c-*myb* and probably NFkB (11). Conversely, endothelin-1 inhibits the growth of stellate cells (12). However, little is known about the signal transduction pathways and the nuclear factors involved in either the normal, quiescent or the activated, proliferating phenotype of hepatic stellate cells. Because stimulation of the cyclic adenosine 3′,5′ monophosphate (cAMP)-dependent protein kinase (PKA)[1] inhibits proliferation of some tumoral cell lines (13–15), we analyzed the potential role of this signal transduction pathway on stellate cell proliferation.

In this study, we found that stimulation of the cAMP/PKA signal transduction pathway (16), or its parallel pathway Ca^{2+}/calmodulin kinase-II (CAMK-II) (17, 18), inhibits proliferation of activated stellate cells. Moreover, our results suggest that activation of either PKA or CAMK-II induces phosphorylation of CREB on Ser 133, which is an important modulator of the stellate cell cycle.

Methods

Cell cultures. Stellate cells were prepared from male Sprague-Dawley rats (400–500 g) by in situ perfusion and single-step density Nycodenz gradient (Accurate Chemical & Scientific Corp., Westbury, NY), as described previously (6, 19). Cells were plated on collagen type I, EHS matrix (Matrigel), or plastic (according to the experimental design) tissue culture dishes, with the initial seeding of fat-storing cells at a density of $2 \times 10^5/cm^2$. Matrigel's (Collaborative Biomedical Products, Bedford, MA) major components are laminin, collagen IV, proteoglycans, entactin, and nidogen. It also contains

1. *Abbreviations used in this paper:* CAMK-II, calmodulin kinase-II; CBP, CREB binding protein; MDA, malondialdehyde; PCNA, proliferating cell nuclear antigen; PKA, cAMP-dependent protein kinase; RARs, retinoic acid receptors.

TGFβ, fibroblast growth factor, and tissue plasminogen activator. Medium was changed every 48 h for all conditions. Stellate cells were identified by their typical autofluorescence at 328 nm excitation wavelength, staining of lipid droplets by oil red, and immunohistochemistry with a monoclonal antibody against desmin (20). Greater than 95% of the cells were stellate cells. Freshly isolated stellate cells were transfected with the mammalian vectors expressing the protein of interest using lipofectin (GIBCO BRL, Gaithersburg, MD) as described by the manufacturer. To increase the transfectability of activated cells, a transfection-enhancing reagent (Life Technologies Inc., Gaithersburg, MD) was added in conjunction with lipofectamine as recommended by the manufacturer. The total amount of transfected DNA was 2.5 μg. The transfection efficiency was 44±10% for day 0 quiescent cells and 28±8% for activated cells growing on a collagen type I matrix. Cells were fixed at 48 or 120 h after transfection for passage 1 and passage 0, respectively. In some experiments, cells were labeled with 2 μCi [^{3}H]thymidine (70–80 Ci/mmol; Amersham Corp., Arlington Heights, IL). After 3 h of labeling, cells were harvested and [^{3}H]thymidine incorporation into DNA was determined as described (21).

Nuclear extract preparation. Nuclei were prepared by a modification of the procedure described previously (21–23). Cells were homogenized in 1 ml of 100 mM KCl, 10 mM Tris, pH 7.6, 1 mM EDTA, 0.5% NP-40, 10 mM NaF, and 10 mM Na pyrophosphate with a glass Dounce homogenizer with a loose fitting pestle. The homogenized cells were placed above a cushion consisting of 2 M sucrose. The nuclei were precipitated by a 4,000 *g* centrifugation at 4°C for 30 min and frozen at −70°C.

Immunohistochemistry. Cells were fixed with acetone/methanol (50:50) at −20°C for 20 min and immunostained as described previously (20, 21). Antibodies directed against β-galactosidase, CREB, CREB-PSer133, or proliferating cell nuclear antigen (PCNA) were obtained from 5 Prime–3 Prime, Inc. (Boulder, CO), New England Biolabs Inc. (Beverly, MA), Upstate Biotechnology, Inc. (Lake Placid, NY), and Novocastra (Burlingame, CA). Fluorescent labels were visualized using a dual channel Zeiss microscope as described previously (21). Cytochromes used were FITC and Texas red (Vector Labs, Inc., Burlingame, CA). The number of PCNA(+) cells was expressed as a percentage of total transfected cells (β-galactosidase [+]). At least 200 cells were analyzed per each experimental point, and a minimum of two observers analyzed each immunohistochemical experiment as described previously (21). Negative control samples were processed in parallel under the same conditions, but with omission of the first antibody. Detection of CREB and CREB-PSer133 in nuclear extracts from rat stellate cells in primary culture or freshly isolated from rat livers was performed by Western blot following the chemiluminescence protocol (DuPont, Wilmington, DE), using antibodies against CREB (Santa Cruz Biotechnologies, Santa Cruz, CA) or CREB-PSer133 (Upstate Biotechnology, Inc.) as described (22, 23).

Statistical analysis. Results were expressed as mean of at least three independent experiments. Both the ANOVA and Bonferroni tests were used for analysis of variance, with a P value of < 0.05 as significant.

Results

First, we examined the role of cAMP on the proliferation of activated primary rat stellate cells. Inhibition of stellate cell proliferation was attempted in passage 0 cells activated on collagen type I for 6 d or in passage 1 cells activated on plastic for 11 d (7, 8, 11). These cells exhibited a high entry into S-phase (> 80%) in agreement with previous studies (11). Cell proliferation was determined by incorporation of [^{3}H]thymidine into DNA, which occurs in S-phase, as described previously (21). As shown in Fig. 1, proliferation of stellate cells whether activated by collagen type I or plastic was blocked by elevation of cAMP with 8Br-cAMP (100 μM), a cAMP isomer resistant

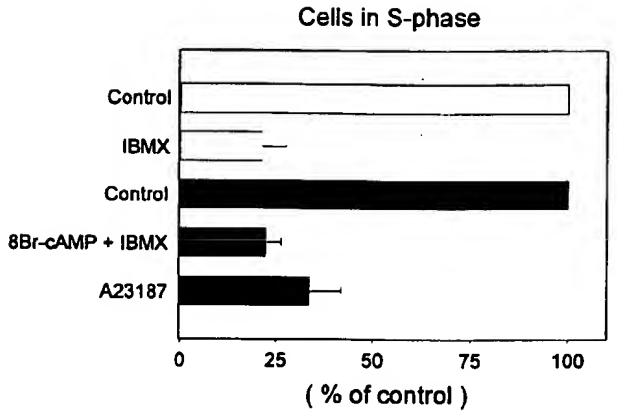

Figure 1. cAMP and Ca^{2+} fluxes inhibit hepatic stellate cell proliferation. The incorporation of [^{3}H]thymidine into DNA was analyzed in primary stellate cells. Cells were cultured on collagen type I (*closed bars*) or on plastic (*open bars*) in media with 10% fetal calf serum. Cells received no treatment (control) or were treated for 24 h with 8Br-cAMP (100 μM), IBMX (500 μM), or A-23187 (1 μM) as described in Methods. [^{3}H]Thymidine (2 μCi) was added for the last 3 h of the incubations. Values represent the percentage of [^{3}H]thymidine incorporation of control cells; $P < 0.05$ for IBMX, 8Br-cAMP + IBMX, and A-23187 compared with controls.

to hydrolysis, and/or isomethylbutyl xanthine (500 μM), a phosphodiesterase inhibitor.

Because intracellular Ca^{2+} fluxes modulate many cellular functions in parallel to cAMP (16–18), we analyzed whether A-23187 (1 μM), an inducer of Ca^{2+} fluxes (24), had similar effects to cAMP on stellate cell proliferation. We found that treatment of activated stellate cells with A-23187 also inhibited cell entry into S-phase (Fig. 1).

Many of the cellular effects resulting from the activation of cAMP/PKA pathway are mediated by the phosphorylation of CREB on Ser 133 (25, 26). Therefore, we assessed the role of CREB-PSer133 on stellate cell cycle. Nuclei were obtained from quiescent and activated stellate cells through a sucrose gradient in the presence of protease and phosphatase inhibitors as described (23, 27). Equal amounts of nuclear protein from quiescent and activated stellate cells were analyzed by SDS-PAGE and visualized by Coomassie blue staining (Fig. 2). Using specific antibodies in a protein immunoblot, we detected CREB as 43-kD monomers in nuclear extracts from quiescent cells. The amount of CREB was decreased in nuclear extracts from proliferating, activated stellate cells (Fig. 2). In nuclear extracts from quiescent cells, CREB was phosphorylated on Ser 133 (Fig. 2). In addition to recognizing CREB in protein immunoblots, anti–CREB-PSer133 detected small quantities of two other proteins after longer exposures. These are most likely members of the CREB-ATF family, ATF1 or CREBβ (38 kD) and CREM (30 kD), that have phosphoacceptor sequences similar to that of CREB-PSer133 (28). The phosphorylation of ATF-1 and CREM can be induced by cAMP and Ca^{2+} (18). Nuclear extracts from activated stellate cells contained negligible amounts of CREB-PSer133 (Fig. 2), suggesting a role for CREB/CREB-PSer133 on the stellate cell cycle. As expected, PCNA, an index of S-phase (29), was present in nuclear extracts from activated

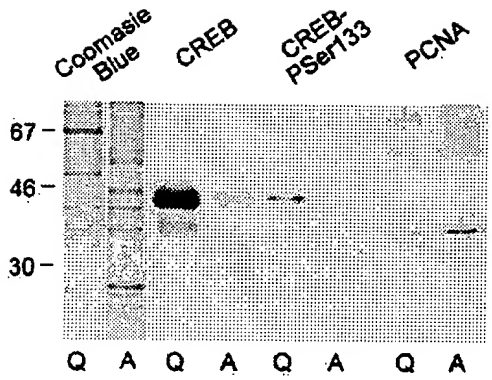

Figure 2. CREB and CREB-PSer133 are expressed in quiescent stellate cells. Protein immunoblots were performed with nuclear extracts (10 μg) from quiescent (*Q*) and activated (*A*) stellate cells as described in Methods, using antibodies against CREB, CREB-PSer133, or PCNA as indicated. Coomassie blue staining of nuclear extracts shows the protein pattern. Molecular markers (kD) are shown.

cells, but not in nuclear extract from quiescent cells (Fig. 2), indicating that the decrease in CREB/CREB-PSer133 in activated cells was not due to a spurious effect of nuclear extraction. Moreover, the results shown in Fig. 2 are representative of four independent nuclear extracts from quiescent and activated stellate cells.

In subsequent experiments, we analyzed whether phosphorylation of CREB on Ser 133 is critical for the inhibition of stellate cell proliferation. Activated cells were transfected with vectors expressing the protein of interest (21, 22), and cultured on collagen type I to maintain their activated phenotype (11). Most of the cells expressing the control β-galactosidase alone entered S-phase (> 80%), as indicated by the expression of PCNA (cyclin; polymerase δ accessory protein) (29) (Fig. 3 *A*). Cells underwent organic fixation which permits the detection of PCNA in the nucleus during S-phase (30). Of interest, coexpression of CREB and a constitutively active PKA (25, 31) together with β-galactosidase inhibited the proliferation of these activated cells. In contrast, expression of β-galactosidase with CREB alone, PKA alone, or CREB-Ala 133 (which lacks the Ser 133 phosphoacceptor [25, 26]) with PKA did not affect stellate cell proliferation (Fig. 3 *A*). These results suggest that PKA-induced phosphorylation of CREB on Ser 133 is necessary for the inhibition of stellate cell proliferation, since unlike wt CREB, CREB-Ala 133 was refractory to the effects of PKA on cell entry into S-phase. Neither the expression of CREB nor the expression of a constitutively active PKA was sufficient to prevent cells to enter S-phase. In Fig. 4, representative examples of the dissociation between CREB/PKA expression and stellate cell cycle S-phase, assessed by PCNA (bright green-yellow), are shown. The transfected cells were identified by the expression of cytoplasmic β-galactosidase (in red). Nuclear expression of PCNA was high in control activated cells but it was markedly inhibited in stellate cells expressing both CREB and PKA. However, the nuclear expression of PCNA in cells transfected with CREB-Ala133 and PKA or CREB and an inactive PKA mutant (25) was comparable with control cells (Fig. 4). Because CAMK-II is activated by Ca^{2+} fluxes and under some experimental conditions is capable of inducing phosphorylation of CREB on Ser 133 (17), we also analyzed the effects of this pathway on the stellate cell cycle. Coexpression of CREB and a constitutively active CAMK-II (32) inhibited proliferation of activated cells (Fig. 3 *B*). In contrast, expression of CAMK-II alone, CREB-Ala133 with CAMK-II, or CREB with an inactive CAMK-II mutant (32) did not alter the stellate cell cycle. Taken together, these results indicate that phosphorylation of CREB on Ser 133 by cAMP/PKA or Ca^{2+}/CAMK-II pathways arrests the stellate cell cycle.

To analyze further the role of CREB-PSer133 on the stellate cell cycle, we performed immunofluorescence for CREB-PSer133 in activated cells using specific antibodies that do not cross-react with nonphosphorylated CREB at Ser 133 (28). As depicted in Fig. 5, when proliferating, activated stellate cells were treated with 8Br-cAMP, IBMX, forskolin (not shown),

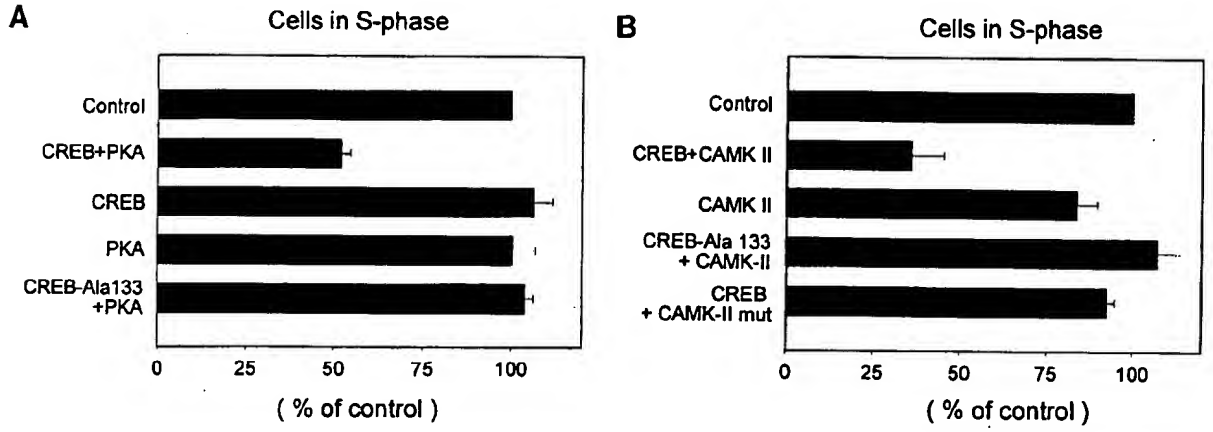

Figure 3. CREB-PSer133 inhibits stellate cell entry into S-phase. Activated stellate cells were transfected with vectors expressing β-galactosidase alone (control) or together with vectors expressing CREB, CREB-Ala133, PKA (C-subunit plasmid MtC) (*A*), CREB, CREB-Ala 133, CAMK-II, or CAMK-II inactive mutant (M42) (*B*) as indicated. Cells were immunostained for β-galactosidase and PCNA 48 h after transfection. Values represent the percentages of cells expressing the transfected DNA that were also in S-phase, setting the percentage for control cells at 100%; $P < 0.05$ for CREB + PKA and CREB + CAMK-II compared with controls.

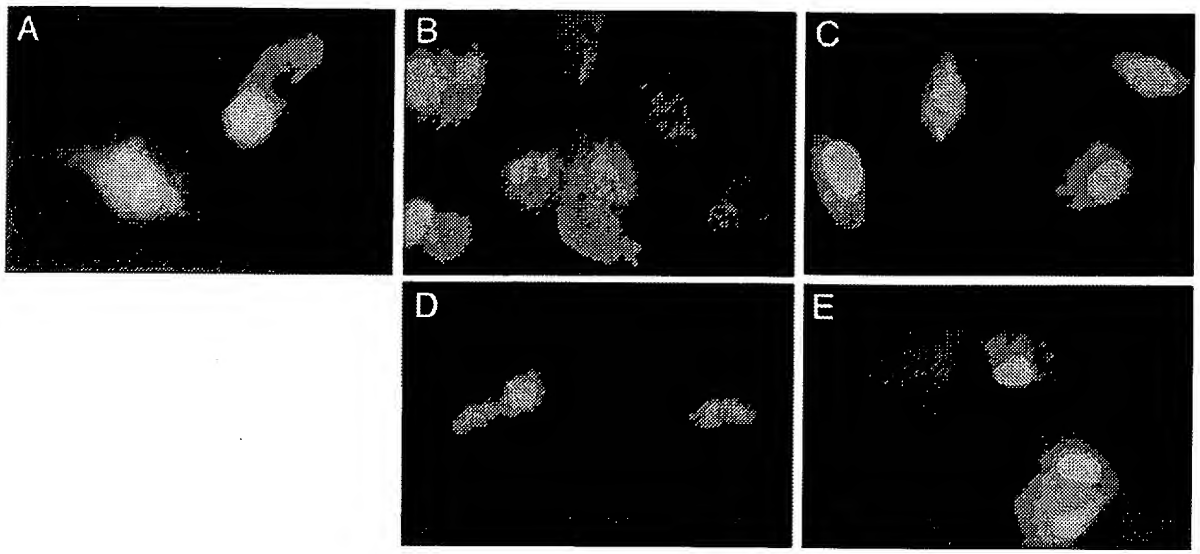

Figure 4. CREB phosphorylation on Ser133 is associated with arrest of the hepatic stellate cell cycle. Activated stellate cells were transfected as described in Fig. 3 with vectors expressing CREB alone (*A*); CREB + PKA (*B*); CREB + PKA inactive mutant (pCaK72M) (*C*); CREB + CAMK-II (*D*); and CREB + CAMK-II mutant (*E*). Cells were fixed 48 h after transfection and dual immunofluorescence was performed for β-galactosidase (red) and PCNA (bright green-yellow). Cells expressing CREB + PKA or CREB + CAMK-II did not enter S-phase.

or A-23187 they expressed nuclear CREB-PSer133, whereas untreated activated stellate cells did not.

Next, we studied whether CREB/CREB-PSer133 contribute to the inhibition of the cell cycle in quiescent stellate cells. In these cells, expression of the *trans*-dominant negative CREB-Ala133 induced a greater than fivefold entry into S-phase of quiescent stellate cells, compared with control cells expressing either β-galactosidase alone or with wt CREB (Fig. 6). The mutant CREB-Ala133 lacks the Ser 133 phosphoacceptor but competes with wt CREB for cognate DNA binding sites, and behaves as an antagonist (25). Thus, expression of a CREB mutant that cannot be phosphorylated on Ser 133 is sufficient to stimulate stellate cell proliferation.

Because oxidative stress and malondialdehyde (MDA), a product of lipid peroxidation, are strong inducers of stellate cell proliferation (11), we tested whether these effects of MDA could be neutralized by CREB-PSer133. We found that day 6 stellate cells cultured on a collagen type I matrix and expressing wt CREB and active PKA proliferated as much as control cells when treated with MDA (Fig. 7). Conversely, butylated hydroxytoluene, an antioxidant that prevents the entry of stellate cells into S-phase induced by collagen type I matrix or TGFα (11), also blocked the proliferation of stellate cells cultured on EHS matrix, when stimulated by the expression of the mutant CREB-Ala133. These results suggest that oxidative stress affects the cascade leading to stellate cell proliferation at a site distal to the effects of CREB-PSer133.

Discussion

Although overproduction of collagen type I by activated hepatic stellate cells is a critical step in the development of liver cirrhosis (4, 5, 7, 8), the mechanisms responsible for the proliferation and activation of hepatic stellate cells remain unclear (3). In this study, we have characterized some of the signal transduction pathways that are involved in the modulation of the stellate cell cycle. Induction of either the cAMP/PKA or Ca^{2+}/CAMK-II pathway (16–18) inhibits proliferation of acti-

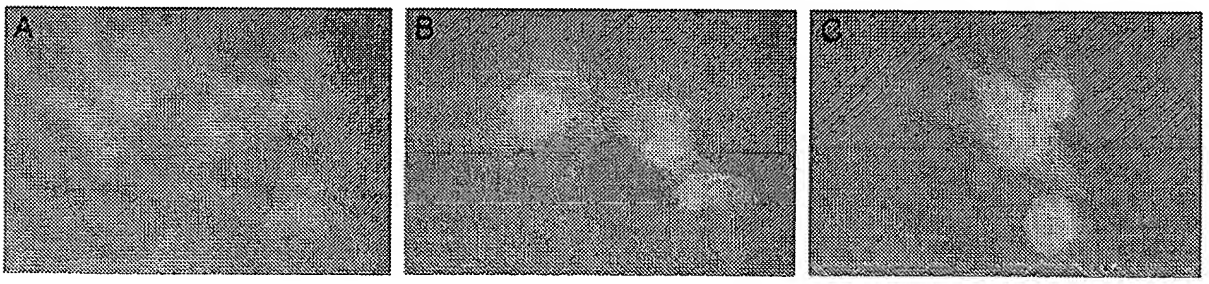

Figure 5. CREB-PSer133 expression is induced in hepatic stellate cells by cAMP or Ca^{2+} fluxes. CREB-PSer133 was detected using specific antibodies and fluorescein-labeled second antibodies. CREB-PSer133 immunofluorescence is shown for activated stellate cells control (*A*); 500 μM IBMX (*B*); and 1 μM A-23187 (*C*) as described in Fig. 1. Cells treated with IBMX or A-23187 expressed nuclear CREB-PSer133.

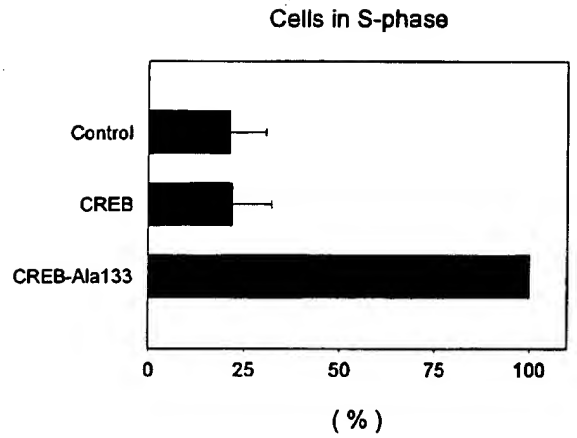

Figure 6. Expression of the *trans*-dominant negative CREB-Ala133 induces stellate cell proliferation. Quiescent primary stellate cells were transfected with vectors expressing β-galactosidase alone, or with vectors expressing CREB or CREB-Ala133 for 120 h. Transfected cells were immunostained for β-galactosidase, and S-phase was determined by the expression of PCNA. Values represent the percentages of cells expressing the transfected DNA that were also in S-phase, setting the percentage for CREB-Ala133 cells at 100%. $P <$ 0.05 for CREB-Ala133 compared with controls.

vated stellate cells. Moreover, we have identified a molecular mechanism leading to entry of stellate cells into S-phase or arrest before the G_1/S boundary.

Our results suggest an important role of CREB Ser133 phosphorylation on the stellate cell cycle. The stellate cell cycle arrest induced by CREB-PSer133 results from the interaction between wt CREB and either active PKA or active CAMK-II, as strongly suggested by the ineffectiveness of: (*a*) CREB-Ala133 in the presence of constitutively active PKA or CAMK-II; (*b*) active PKA or active CAMK-II; and (*c*) wt CREB in the presence of mutant PKA or mutant CAMK-II. Also, we determined that quiescent cells can be induced to enter S-phase when they expressed the *trans*-dominant negative CREB-Ala133. In agreement with these results, we found that stellate cell cycle can be arrested by elevation of cAMP with 8Br-cAMP or IBMX or by inducing Ca^{2+} fluxes with the Ca^{2+} ionophore A-23187.

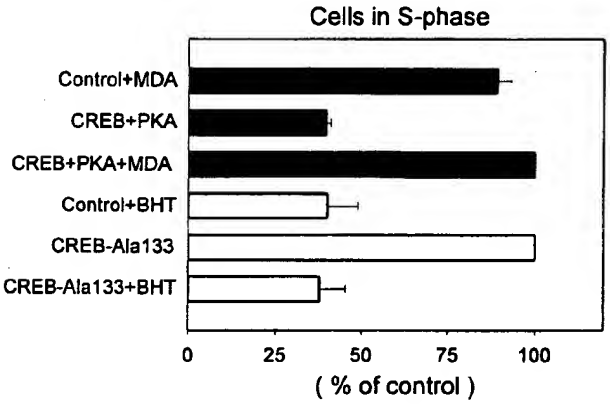

Figure 7. Oxidative stress affects the stellate cell cycle independently of CREB-PSer133. Stellate cells were cultured on collagen type I matrix (*closed bars*) or EHS (*open bars*), respectively. Cells were transfected with expression vectors and treated for 120 h with MDA (200 μM), or for 48 h with butylated hydroxytoluene (*BHT*) (50 μM) as indicated. Cells were stained as described in Fig. 4. Values represent the percentages of cells expressing the transfected DNA that were also in S-phase setting the percentage for MDA (*closed bars*) and CREB-Ala133 (*open bars*) at a 100%. $P < 0.05$ for CREB + PKA compared with CREB + PKA + MDA; and CREB-Ala133 compared with CREB-Ala133 + BHT.

In contrast, quiescent stellate cell entry into S-phase is markedly induced by interfering with the activity of CREB/CREB-PSer133, with the *trans*-dominant negative CREB-Ala133. As for many other cellular functions (33), intracellular Ca^{2+} as well as cAMP seems to modulate the stellate cell cycle. In addition, activation of the cAMP/PKA pathway by M-phase–promoting factor is required for the transition from mitosis to interphase (34). Interestingly, either increased cAMP (35) or Ca^{2+} mobilization (24) inhibits collagen production by fibroblasts. A similar inhibitory effect of cAMP or Ca^{2+} fluxes on collagen production by stellate cells is expected, since quiescent stellate cells have a low expression of collagen type I gene compared with their activated, proliferating counterparts (4, 5, 19).

Because oxidative stress and reactive aldehydes induce the proliferation of quiescent stellate cells (11), and stimulate collagen transcription (36, 37), and antioxidants suppress the proliferation of activated stellate cells (11) as well as collagen gene expression (38), we analyzed whether oxidative stress affects the cell cycle at a site proximal or distal to the effects of CREB-PSer133. The antioxidant butylated hydroxytoluene (38) prevented the stimulation of quiescent stellate cell entry into S-phase by the mutant CREB-Ala133 (an antagonist of CREB). Moreover, exposure of proliferating, activated stellate cells to MDA, an aldehyde product of lipid peroxidation (37), prevented the cell cycle arrest by wt CREB and active PKA. Collectively, these results suggest that oxidative stress modulates the stellate cell cycle cascade at a site distal to the effects of CREB-PSer133. In this context, we have also demonstrated that quiescent stellate cells express nuclear CREB and CREB-PSer133, whereas nuclear expression of CREB-PSer133 in activated stellate cells is stimulated by the elevation of cAMP or by Ca^{2+} mobilization. Given the results of this study, we expect that stellate cell proliferation will be facilitated by a targeted disruption of the CREB gene (39). In contrast to CREB-PSer133, CREB phosphorylation on Ser 119 seems to be required for T cell activation and cell-cycle progression (40).

Although little is known about the mechanisms by which CREB/CREB-PSer133 affects the cell cycle, CREB/CREB-PSer133 function can be modulated by CREB binding protein (CBP) (41, 42). Recent studies indicate that CBP binding affinity can be regulated by site-specific phosphorylations mediated by PKA (16) and $p90^{rsk}$ (43). The precise molecular interaction of CREB/CREB-PSer133 with CBP and other transcription factors is poorly understood. However, Kamei et al. (44) have proposed that CBP could act as a key integrator of cellular functions by selectively interacting with different transcription factors. For example, CBP is known to bind preferentially to the oncoproteins c-*myb* and c-*jun*, or to the cell-cycle arresting factors RARs (retinoic acid receptors) and CREB, according to the biological conditions (41–45). Taken together, these results suggest the possibility that CREB/CREB-PSer133 could compete with c-*myb* for interaction with CBP (45), thereby af-

fecting the role of c-*myb* in the activation of stellate cells (11). Likewise, the important role of retinoids on quiescent stellate cell phenotype (7, 9) could be related to their activation of RARs and the potential facilitation of the interaction between CBP and CREB-PSer133 (41, 42). Alternatively or in addition, the role of cAMP may reflect its modulation of the retinoic acid–dependent RAR's transcription, given the presence of CREB binding sites on the RAR-β_2 promoter (46).

In this study, we have identified a novel function of CREB-PSer133 as a regulator of the cell cycle in highly differentiated cells. A similar inhibitory role on the cell cycle has also been found for other transcription factors such as MyoD, C/EBPα, and LAP (21, 47, 48), known to induce differentiated phenotypes in skeletal muscle, adipocytes, and hepatocytes (49). However, in poorly differentiated cell lines, such as erythroleukemia K562 and pheochromocytoma PC-12, growth factors activate the RSK2 kinase which induces phosphorylation of CREB on Ser 133 (50), indicating that under certain growth conditions, CREB-PSer133 is not sufficient to block cell cycle progression, as suggested by our results with oxidative stress (Fig. 7). In summary, our study provides insights into the molecular mechanisms modulating hepatic stellate cell cycle, as well as a rationale for potential therapeutic approaches for hepatic fibrosis.

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Inducible cAMP early repressor ICER down-regulation of CREB gene expression in Sertoli cells

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Abstract

The cAMP response element binding protein (CREB) and the cAMP-responsive element modulator (CREM) are cyclically expressed in the seminiferous tubules during spermatogenesis. In the somatic Sertoli cells, which are the major supporters of germ cell development in the seminiferous tubules, the expression of CREB is cyclical and appears to be regulated by the levels of cAMP produced in response to the pituitary derived follicle-stimulating hormone FSH. Cyclic AMP response elements (CREs) located in the promoter of the CREB gene were shown earlier to be implicated in an autopositive feedback loop that up-regulates the expression of CREB. Here we show that in Sertoli cells FSH-mediated induction of the CREM repressor isoform, ICER (inducible cAMP early repressor) is correlated with the inhibition and delay of CREB gene expression in the seminiferous tubules. ICER binds to the two CREs located in the promoter of the CREB gene and in transient transfection assays of Sertoli cells, ICER expression vectors down-regulate transcription of a reporter gene driven by the CREB gene promoter. In addition, analyses of ICER and CREB gene expression in isolated segments of rat seminiferous tubules reveals stage-specific and cycle-dependent expression of ICER. The periods of enhanced expression of ICER correspond to the stages of spermatogenesis with the lowest levels of CREB expression. We suggest that the expression of ICER in Sertoli cells may contribute to the periodic repression of CREB gene expression during the repeated 12-day cycles of spermatogenesis, and may be required to reset the levels of activator CREB prior to the initiation of each new cycle of spermatogenesis.

Keywords: CREB; ICER; Sertoli cell; Follicle stimulating hormone; Spermatogenesis; Testis; Gene expression

1. Introduction

CREB (cAMP response element binding protein) and CREM (cAMP response element modulator) are cAMP-responsive members of the bZIP family of transcription factors. They are so named because of their highly conserved DNA-binding domains consisting of an amino-proximal DNA-recognition basic region (b) and a carboxyl-terminal dimerization domain (ZIP, leucine zipper) (Hoeffler and Habener, 1990; McKnight, 1991; Meyer and Habener, 1993; Habener et al., 1995). CREB and CREM (and activating transcription factor-1, ATF-1) are distinguished amongst the bZIP proteins by the marked responsivity of their transcriptional transactivation functions to phosphorylation by cAMP-dependent protein kinase A (Meyer and Habener, 1993; Habener et al., 1995). These bZIP proteins bind to specific cAMP response elements (CREs) located in the promoters of cAMP-responsive genes and activate gene transcription in response to

Abbreviations: CREB, cAMP response element binding protein; CREM, cAMP response element modulator; ICER, inducible cAMP early repressor; EMSA, electrophoretic mobility shift assay; FSH, follicle-stimulating hormone; bZIP, basic region leucine zipper; DBDI, II, DNA-binding domains I or II of CREM..

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phosphorylation (Meyer and Habener, 1993; Habener et al., 1995). Both CREB and CREM are expressed at high levels in the testis and their expression is controlled by cAMP signalling mediated by the interactions of the pituitary gonadotropic hormone, FSH with stimulatory G-protein-coupled receptors found on the somatic Sertoli cells, (Steinberger et al., 1978; Waeber et al., 1991; Foulkes et al., 1993).

In Sertoli cells CREB mRNA levels are transiently induced in a repeated cyclical pattern corresponding to the specific 12-day temporal and anatomical cell association stages of spermatogenesis (Waeber et al., 1991). Levels of CREB mRNA increase in cell association stages II–V (Waeber et al., 1991), after some delay, following increases in FSH-induced cAMP levels in stages XII–V (Kangasniemi et al., 1990; Walker and Habener, 1996). CREB mRNA levels then fall rapidly to nearly undetectable levels in stages VII–XIV as cAMP levels decrease due to internalization of FSH receptors and the down-regulation of the FSH receptor (Themmen et al., 1991). The characterization of the promoter of the CREB gene identified cAMP response elements (CREs) that contribute to the cAMP induction of the transcription of the CREB gene (Meyer et al., 1993; Meyer and Habener, 1993). Subsequent studies showed that phosphorylation of CREB bound to a CRE by cAMP-dependent PKA stimulates CREB gene transcription and the consequent production of additional CREB, indicating the existence of an autopositive feedback loop (Walker et al., 1995). This autopositive regulation of CREB gene expression is proposed to account for the large increase in CREB mRNA levels that accumulate in the nuclei of Sertoli cells during stages II–VI of the spermatogenic cycle (Waeber et al., 1991). However, the delay in initiation of the positive feedback of the CREB gene until well after cAMP levels initially rise in stage XII has remained unexplained.

ICER, the cAMP-responsive repressor form of CREM is one candidate regulator of the CREB gene in Sertoli cells. Transcription of the ICER is also autoregulated by cAMP signalling as an internal promoter (P2) located in the 3′ region of the CREM gene is activated by cAMP (Molina et al., 1993). cAMP-responsive CREB and CREB-like activator proteins interact with four CAREs (cAMP autoregulatory response elements) in the P2 promoter to stimulate transcription (Molina et al., 1993). The mRNA that encodes the ICER repressor consists of a short ICER-specific region followed by the bZIP DNA-binding domain (Molina et al., 1993).

Earlier we reported that the autopositive upregulation of CREB gene expression in testicular germ cells is interrupted by the switch in expression from activator to inhibitor CREBs (I-CREBs) (Girardet et al., 1996; Walker et al., 1996; Walker and Habener, 1996). However, I-CREBs appear to be expressed at low levels in the somatic Sertoli cells leaving unanswered the mechanisms by which the cyclical upregulation of CREB is interrupted in Sertoli cells (Walker and Habener, 1996). Recently, it was reported that the ICER repressor is expressed in primary rat Sertoli cells in response to FSH and is proposed to down regulate the transcription of the FSH receptor gene in these cells (Monaco et al., 1995). Here we report findings that FSH-induced expression of ICER down regulates the expression of the CREB gene in primary rat Sertoli cells and thereby provides a potential explanation for the cyclical fluctuations in CREB gene expression during spermatogenesis specifically in Sertoli cells. Further, we show by studies of isolated rat seminiferous tubules that CREB and ICER are expressed reciprocally at different stages of the 12-day cycle of spermatogenesis.

2. Experimental procedures

2.1. Isolation of Sertoli cells, seminiferous tubules and preparation of protein extracts

Sertoli cells isolated from 16-day Sprague–Dawley rat testis after collagenase and trypsin digestion (Walker et al., 1995) were cultured on matrigel coated plates (Collaborative Research, Bedford, MA) in serum free medium containing 50% Dulbecco's modified Eagle's medium, 50% Ham's F-12. Media was supplemented with 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 1 μM retinoic acid, 10 ng ml^{-1} epidermal growth factor, 3 μg ml^{-1} cytosine β-D-arabinofuranoside, 2 mM glutamine, 1 mM sodium pyruvate, 100 u ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Walker et al., 1995). Sertoli cells were routinely greater than 95% pure as determined by phase microscopy and alkaline phosphatase staining (Chapin et al., 1987). Animal studies were conducted in accordance with the principles and procedures outlined in 'Guidelines for Care and Use of Experimental Animals'.

Sertoli cells (1×10^9 cells) were collected after 0.5–24 h of stimulation with 100 ng ml^{-1} FSH (ovine pituitary FSH, Sigma, St. Louis) and 0.5 mM 3-isobutyl-1 methylxanthine (IBMX) or empty vehicle. Nuclear extracts of Sertoli cells were prepared as described (Schreiber et al., 1989). Seminiferous tubules were isolated from 60-day Sprague Dawley rats and maintained in enriched Krebs–Ringer bicarbonate buffer (Bellvé et al., 1977). The stage dependent transillumination pattern was identified using a stereomicroscope and 2 mm sections were sequentially cut and flash frozen beginning with the stage VIII | IX border identified by the distinct darklight interface (Toppari and Parvinen, 1985, Kangasniemi et al., 1990). Whole cell protein extracts were prepared from the tubule sections by shaking the tissue for 15 min in ELB buffer (250 mM

NaCl, 0.1% NP40, 50 mM Hepes pH 7.0, 5 mM EDTA, 0.5 mM DTT, and protease inhibitor cocktail) at 4°C (Walker, et al., 1992), followed by centrifugation (12000 × g) for 5 min to remove cellular debris. Protein concentrations were determined using the BioRad Protein Assay.

2.2. DNA-binding assays

Electrophoretic mobility shifts (EMSA) binding reactions were performed in the presence of 1 μg poly (dI–dC) using 5 μg of protein extract as described (Deutsch et al., 1988) and ^{32}P-labeled oligonucleotide probes containing either a consensus CRE (COLCRE) (5′-GATCCGGCTGACGTCATGAAGCTAGATC-3′, or the wild type CRE1 and CRE2 region of the CREB promoter (CREBCRE) (5′-GATCCGTTGGTGAG TGACGCGGCGGAGGTGTAGTTTGACGCGGTG TGAG-3′). For immune supershift assays, extracts from Cos-1 cells expressing ICER were incubated with the ^{32}P-labeled oligonucleotide probes in the presence of rabbit pre-immune sera, CREMS4 rabbit antisera that recognizes the carboxyl-terminal region of CREM and ICER, guinea pig pre-immune sera or ICER-specific peptide guinea pig antisera. DNA-protein complexes were resolved on 5% nondenaturing polyacrylamide gels and visualized by autoradiography. For competition EMSA experiments to estimate relative DNA-protein affinities, ICER and CREB proteins (2 μl) derived from coupled in vitro transcription-translation reactions (TnT, Promega) were incubated with CREBCRE probe (0.5 pmol) and increasing amounts of unlabeled CREBCRE probe (0.5–50 pmol). The relative levels of DNA-protein complexes were determined by densitometric scanning using the BIO-RAD Molecular Analyst system.

2.3. Construction of ICER expression vectors

The ICERIγ isoform was isolated from rat testis RNA using reverse transcriptase and polymerase chain reaction (PCR). A dT_{17} 3′ oligonucleotide was used to reverse transcribe cDNAs from 5 μg of Sertoli cell RNA. The ICERIγ isoform was amplified by PCR using an oligonucleotide corresponding to the ICER 5′ untranslated region (5′-ACTCTATATGCAAAAAGC CC-3′) and the CREMR5 oligonucleotide (5′-GAGCTCGAATTCCCAATTCACACTCTACAGCA G-3′) corresponding to the DNA-binding domain I (DBDI) region of CREM (exon Ia) (8) located 120 bp downstream of the translation termination codon. ICER cDNAs were subcloned into the PCRII vector (Invitrogen, San Diego) for expression of ICER in vitro. For expression in eukaryotic cells, ICERIγ was excised from the PCRII vector with EcoR1 and inserted into pCMV5 (Chen et al., 1991) linearized with EcoR1.

2.4. Northern blotting

RNA from primary Sertoli cells treated with FSH and IBMX or empty vehicle was prepared with Trizol reagent (Gibco-BRL) according to the manufacturers instructions and 10 μg of each RNA sample was subjected to Northern analysis as described (Walker et al., 1995). Northern blots were probed with ^{32}P labeled ICERIγ or CREB cDNA probes prepared by random priming. Equal loading of RNA samples was confirmed by staining the gel with SyBR Green II or Ethidium Bromide dye. The RNA products on the autoradiograms were scanned with a computing densitometer to provide a semiquantitative evaluation of the relative levels of the RNAs (ImageQuaNT, Molecular Dynamics).

2.5. Immunocytochemistry

Frozen sections from adult rat testis were immunostained with preimmune sera or ICER-specific antisera (rabbit) directed against amino acids 2–11 of ICER (Bodor et al., 1996 PNAS) and the antigen–antibody immune complex was visualized using a Cy3 fluorescent secondary anti-rabbit serum (Jackson Immunoresearch Laboratories) or an anti-rabbit biotinylated antibody and colorimetric staining as described in the Vectastain Elite kit (Vector Laboratories).

2.6. Expression of proteins in bacteria and Cos-1 cells

Synthesis of recombinant proteins was induced in *E. coli* BL21 (DE3) pLysS. CREB and ICER were produced from plasmids containing the appropriate cDNA under the control of the T7 polymerase promoter in the pET-3b prokaryotic expression vector (Vallejo et al., 1992). ICER and CREB were produced in Cos-1 cells transiently transfected with pCMV5 ICER or pCMV5 CREB expression vectors (5 μg) (Seldon 1989). Cos-1 whole cell extracts were prepared 48 h post-transfection after lysis of cells in EIB buffer (Walker et al., 1992).

2.7. Sertoli cell transfections and CAT assays

Primary Sertoli cells were transfected 3 days after isolation as described (Walker et al., 1995), except that 1 μg of CAT reporter plasmids in a total of 5 μg DNA were used per 60 mM dish. The cells were cotransfected with either the wild-type ICERIγ or the mutated ICERmut expression plasmids. The ICERmut has a deletion in the leucine zipper dimerization domain, so it cannot dimerize or bind DNA (Bodor et al., 1995). Cells were harvested 48 h post-transfection and CAT activity determined as described (Walker et al., 1995). To activate CREB, certain transfections included the plasmid pRSVCat-β that expresses the C_β isoform of the cata-

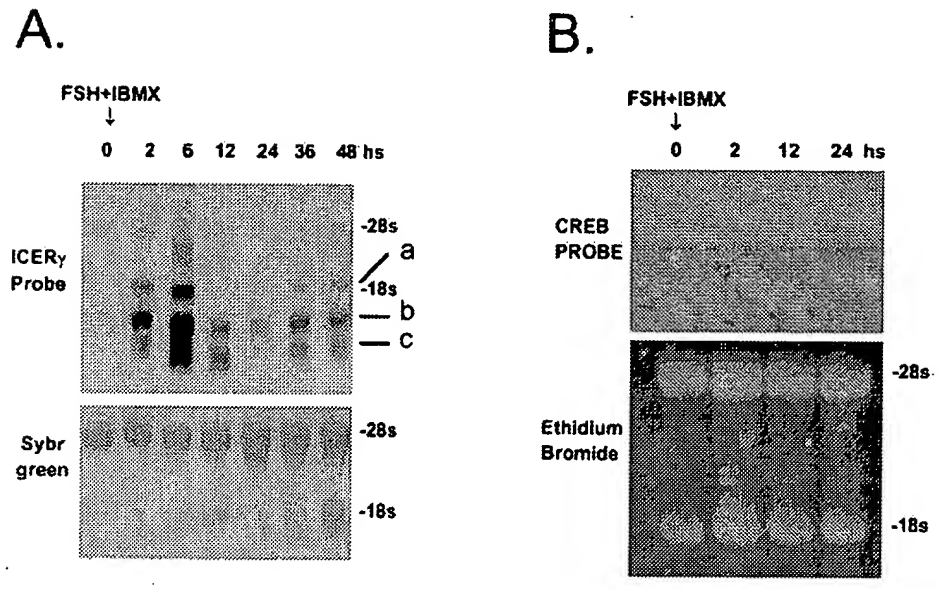


Fig. 1. FSH and IBMX induce ICER and repress CREB mRNAs in primary rat Sertoli cells. (A) Northern blot of RNA isolated from primary Sertoli cells, either untreated (0 h) or stimulated with FSH and IBMX for 2–48 h. Top, ICER mRNA was hybridized with a ^{32}P-labeled RNA derived from ICERIγ cDNA; a, b, and c refer to the three alternatively spliced forms of ICER detected by the ICER specific probe. Bottom, SyBR Green staining of the gel used to characterize ICER mRNA levels showing equal loading of RNA (28S and 18S ribosomal RNA) for all time points. (B) Northern blot of RNA prepared from rat Sertoli cells after their treatment with FSH and IBMX. Membrane-bound RNA was probed with a CREB cDNA probe. Bottom, Ethidium Bromide staining of the gel used to show equal loading of RNA (28S and 18S ribosomal RNA) for all time points.

lytic subunit of protein kinase A (Maurer, 1989). Cells were also transfected with the cAMP responsive pENKAT-12 plasmid as a positive control (Comb et al., 1986). The pENKAT-12 consists of the CAT gene under control of the proenkephalin promoter. Relative CAT activities of the various experimental conditions were normalized to the activities of-278CREBCAT containing 278 bp upstream of the CREB translation start site (Walker et al., 1995) or pENKAT-12 co-transfected with pRSVCat-β which were taken as 100% activity.

3. Results

3.1. ICER and CREB mRNA levels are regulated by FSH and IBMX in primary rat Sertoli cells

To determine the mechanism accounting for the downregulation of the transcriptional expression of the CREB gene in Sertoli cells, the role of ICER in the regulation of the expression of the CREB gene was investigated in primary Sertoli cells treated with FSH and 3-isobutyl-methylxanthine (IBMX) over a time course of 48 h. Measurements of ICER and CREB mRNA levels in extracts of Sertoli cells at increasing times after the addition of FSH and IBMX was taken as an initial index of the effects of cAMP signalling on the expression of the ICER and CREB genes. Time-dependent, cyclical fluctuations in the levels of ICER and CREB mRNAs were observed by Northern RNA blot analyses (Fig. 1). Before the addition of FSH and IBMX, no ICER mRNA was detectable (Fig. 1(A)). By 2 h after the addition of FSH and IBMX, ICER mRNA became readily detectable, increased markedly by 6 h, and then decreased from 12 to 24 h, only to increase again at 36–48 h. In addition, at least three ICER mRNAs were detected that likely correspond to alternatively spliced isoforms of the mRNA (Rauchaud et al., 1997). These observations are typical for the expression of ICER which is itself under cyclical autoregulatory control in response to cAMP signalling. When levels of ICER rise they feed back on the ICER gene promoter to repress it (Molina et al., 1993). In contrast, treatment of Sertoli cells with FSH initially causes a small apparent decrease in CREB mRNA levels. However, in agreement with earlier studies employing 8-Br cAMP as a stimulant (Walker et al., 1995), addition of FSH and IBMX first results in the elevation of CREB mRNA levels between 12 and 24 h (Fig. 1(B)). Because ICER levels are high immediately after FSH-stimulation and the induction of CREB occurs much later, the possibility that ICER was responsible for the repression and delay in CREB gene transcription was investigated.

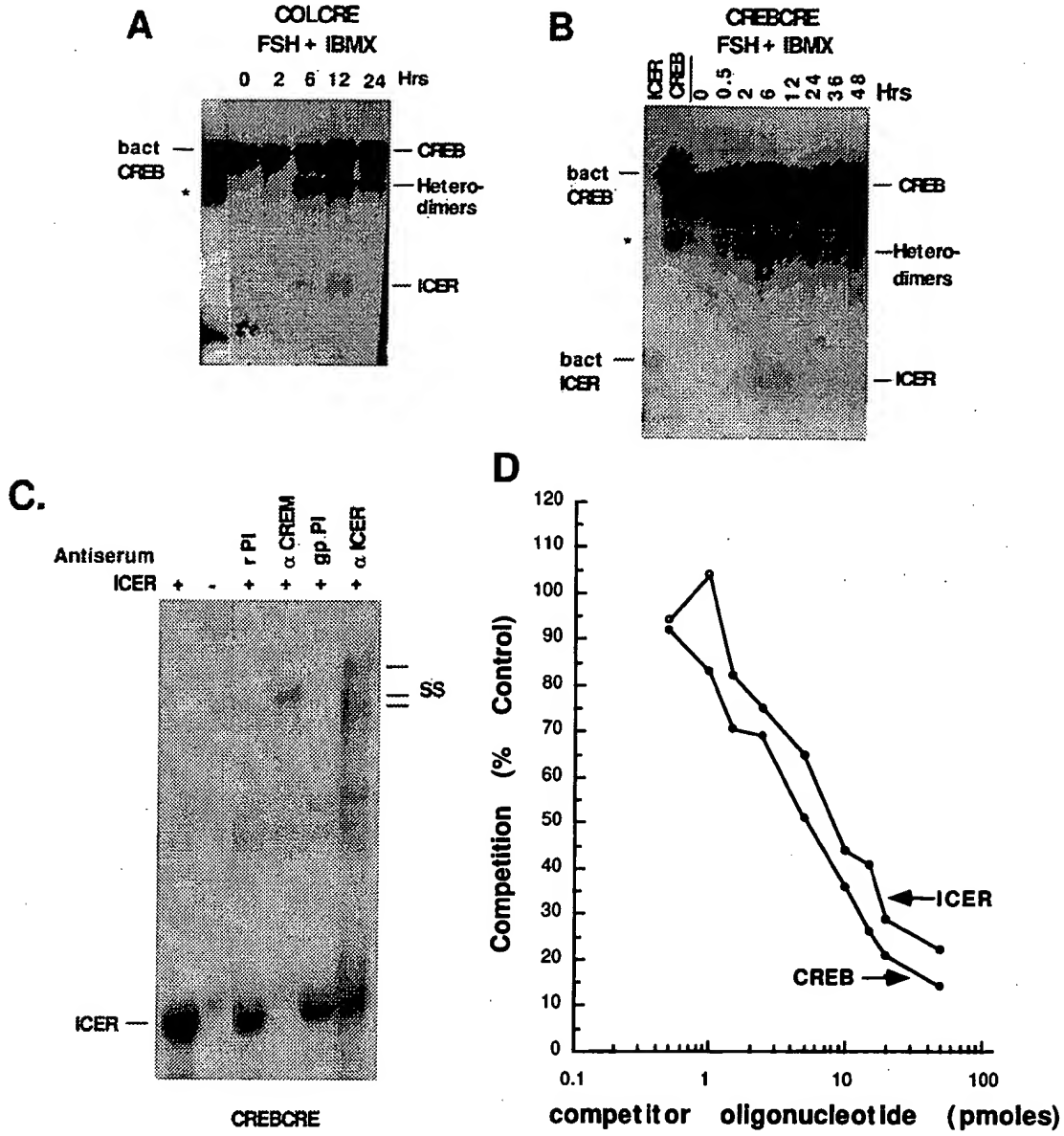


Fig. 2. Electrophoretic mobility gel shift assays showing that FSH and IBMX transiently induces ICER in primary Sertoli cells. (A) A consensus CRE probe (COLCRE) (Deutsch et al., 1988) or (B) a CREB gene promoter CRE (CREBCRE) (Walker et al., 1995) were incubated with bacterially expressed CREB, bacterially expressed ICERIγ or nuclear extracts from primary Sertoli cells, either untreated (0 h) or stimulated with FSH and IBMX for 0.5, 2, 6, 12, 24, 36, or 48 h. CREB, and ICER homodimer-probe and CREB-ICER heterodimer-probe complexes are indicated. Complexes formed with bacterially expressed CREB and ICER are slightly larger due to added vector-encoded amino acids. In A and B an asterisk denotes complexes formed with smaller CREB-like proteins produced by processing of overexpressed CREB in bacteria. (C) extracts from Cos-1 cells transfected with an ICERIγ expression vector were incubated with an oligonucleotide containing the CREB CRE element of the CREB promoter in EMSA analysis. In immunosupershift analyses, rabbit preimmune serum (αrPI), rabbit CREMS4 (αCREM) antiserum directed against the carboxyl-terminus of CREM and ICER, guinea pig preimmune serum (αgp PI), or guinea pig ICER-specific antisera directed against the seven unique amino terminal amino acids of ICER (αICER) were added to the DNA-protein binding reactions. The ICER-probe complex and antiserum-dependent ICER up-shifted complexes (SS) are indicated. (D) competition analysis to determine the relative affinities of CREB and ICER for the CREB promoter CREs. CREB and ICER proteins produced by coupled in vitro transcription-translation reactions were incubated with radiolabeled CREBCRE probe and increasing amounts of unlabeled CREBCRE competitor as shown. The DNA-protein complexes were resolved by nondenaturing PAGE and the intensities of the DNA-protein complexes were quantitated using the BIO-RAD image analysis system. The results shown are an average of four experiments. The standard error was less than 20% for each observation.

3.2. ICER and CREB protein levels are regulated by FSH and IBMX in primary rat Sertoli cells

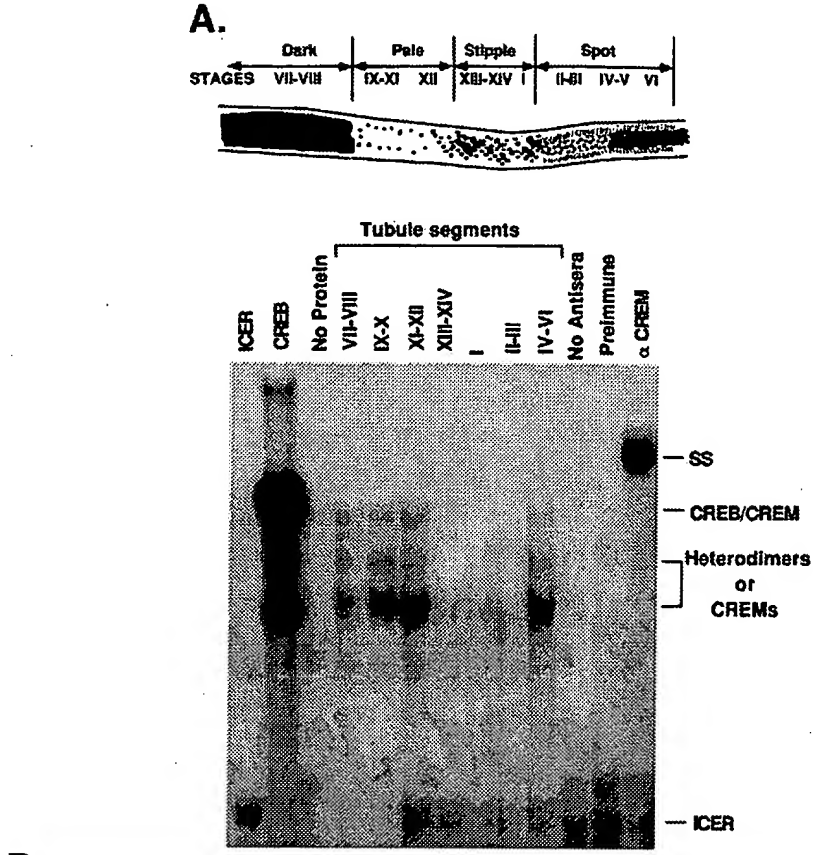
In DNA-binding EMSA studies, nuclear extracts from Sertoli cells treated with FSH and IBMX were incubated with a ^{32}P-labeled oligonucleotide probe containing an optimized symmetrical palindromic CRE (COLCRE) (Fig. 2(A)). By 6 h after stimulation of the Sertoli cells with FSH and IBMX, a CRE-binding protein corresponding to the mobility of ICER was induced. To determine whether the ICER-like protein could bind to the CRE motifs of the CREB promoter, an oligonucleotide consisting of the two tandemly-arranged asymmetrical CREs, as they appear in the context of the promoter of the CREB gene (CREBCRE) (Meyer et al., 1993), was used in binding studies of FSH-treated primary Sertoli extracts (Fig. 2(B)). A DNA-protein complex that migrated close to that observed due to the binding of bacterially expressed ICER protein was induced 6 and 12 h after FSH treatment with levels falling significantly by 24 h at a time when CREB levels begin to rise. Concomitant with the appearance of the faster migrating ICER complex, a new complex appeared intermediate in mobility between those of ICER and CREB. This intermediate complex likely represents heterodimers of ICER and CREB or CREB-like proteins because it migrates in the same position as complexes derived from mixtures of partially purified bacterial extracts of CREB and ICER (data not shown). In fact, the relative intensities of the complexes formed suggests that in Sertoli cells, ICER is more likely to form heterodimers with CREB than it is to form homodimers. The co-regulation of these two ICER-containing complexes are maintained after longer periods of FSH treatment since both CREB/ICER heterodimers and ICER homodimers appear to be deinduced after 24–36 h, reappearing again at 48 h (Fig. 2(B)).

That the inducible ICER complex contained the ICER repressor protein is supported by the findings that the induced DNA-protein complex comigrated with a complex formed by ICER produced in bacteria (Fig. 2(B)), the pattern of induction matched that of ICER RNA produced in Sertoli cells after FSH treatment (Fig. 1(A, B)) and that of FSH-induced ICER protein previously described in Sertoli cells (Monaco et al., 1995). To further confirm that ICER binds to the CREBCRE probe, supershift EMSA analysis was performed using antisera raised either against CREM (αCREM) which recognizes the carboxyl-terminal region of CREM that is shared with ICER or an ICER-specific antiserum (Fig. 2(C)). For this study, extracts from Cos-1 cells transfected with CREB or ICER expression vectors were used in the binding reactions. Both of the ICER antisera, but not pre-immune sera, caused disruption and upshifting of the FSH and IBMX-inducible ICER-containing complex (Fig. 2(C)).

For ICER to be an effective repressor of CREB transcription it must be able to bind to the CREB promoter CRE motifs with affinity similar to that of CREB. This is particularly important because members of the CREB family of transcription factors have been found to bind to asymmetrical CREs, such as those within the CREB promoter, with lower affinity than to symmetrical CREs (Nichols et al., 1992). The relative affinities of ICER and CREB for the CREB promoter CREs were measured using a competitive EMSA approach. These assays were performed with constant inputs of CREB or ICER protein and ^{32}P labeled CREB CRE probe in the presence of increasing amounts of non-labeled CREB CRE probe. The intensities of the DNA-protein complexes were measured using image analysis software (Molecular Analyst, Bio-Rad Laboratories) and competition curves were generated. The relative affinities of each protein for the CREs was estimated from the competitor values at which 50% of the binding was inhibited. The relative affinity of ICER for the CREB promoter CREs was found to be close to that of CREB: 9.1 pmol of CRE CREB competitor probe was required to compete 50% of ICER binding compared to the 5.2 pmol of competitor required to reduce CREB-binding by 50% (Fig. 2(D)). These data support the idea that the FSH-induced increases in ICER expression are capable of competing effectively with CREB for occupancy of the CREs.

3.3. Cyclical expression of ICER and CREB in rat seminiferous tubules in vivo

Although a transient induction of the expression of ICER by FSH has been shown in cultured Sertoli cells in vitro (Monaco et al., 1995), the dynamics of such expression in vivo have not been investigated. Therefore, we examined the temporal pattern of the induction of ICER in Sertoli cells within the context of the intact seminiferous tubule. In the rat, 14 stages of spermatogenesis define specific cell associations of developing germ cells in the seminiferous epithelium (Leblond and Clermont, 1952; Perey et al., 1961). These 14 stages of development appear in succession along the length of seminiferous tubules in 12-day repeating cycles designated as the waves of the seminiferous epithelium (Leblond and Clermont, 1952; Perey et al., 1961). Whole cell extracts prepared from a contiguous series of tubule segments isolated by microdissection and representing the stages of spermatogenesis were analyzed in EMSA studies to determine the stages of spermatogenesis in which ICER is expressed. Incubation of the tubule extracts with a probe containing the CREB promoter CRE motifs (CREBCRE) resulted in the formation of complexes comigrating with those formed in the presence of ICER produced by trans-


A.
Dark
Pale
Stipple
Spot
STAGES VII-VIII IX-XI XII XIII-XIV I II-III IV-V VI
Tubule segments
ICER
CREB
No Protein
VII-VIII
IX-X
XI-XII
XIII-XIV
I
II-III
IV-V
VI
No Antisera
Preimmune
α CREM
SS
CREB/CREM
Heterodimers or CREMs
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ICER
IX
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VII
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Fig. 3.

formed bacteria (Fig. 3(A)). ICER-like binding activity is induced at approximately stage XI–XII with levels falling to basal levels by stages I–II. Interestingly, the induction of ICER-like binding activity corresponds to the initial increase in cAMP levels in Sertoli cells during stages XII–XIV (Kangasniemi et al., 1990).

In the seminiferous tubules of the adult rat, Sertoli cells make up only 3% of the cell population with germ cells accounting for greater than 95% of the cells (Bellvé et al., 1977). In contrast to ICER, which has only been detected in Sertoli cells (Delmas et al., 1993; Molina et al., 1993; Monaco et al., 1995), the production of various CREB and CREM isoforms in germ cells complicates the interpretation of the relative levels of Sertoli-derived CREB present in the stage-specific seminiferous tubule extracts. In addition, due to the low ratio of Sertoli to germ cells in microdissected seminiferous tubules it is technically difficult to purify sufficient numbers of Sertoli cells to directly compare the relative CREB and ICER levels at each stage of spermatogenesis using immunoblot or immunoprecipitation assays. Therefore, to confirm that ICER is induced stage-specifically in Sertoli cells, immunocytochemistry of adult rat testis was performed. Using an ICER-specific antiserum and a flourescent secondary antibody, immunostaining was restricted to nuclei along the basement membrane of the seminiferous tubule (Fig. 3(B)). Periodic acid Scheiff-hematoxalin staining of adjacent serial sections showed that ICER immunostaining was induced in the nuclei of Sertoli cells at stages XI-I (data not shown). Basal levels of ICER were seen in all other stages examined. Further examination of ICER expression using a colorimetric immunocytochemistry assay showed stage-specific staining of Sertoli nuclei with the ICER antisera. The more mature spermatocyte and spermatid germ cells were not stained with the ICER antisera; however, we are unable to rule out the possibility that spermatogonia may express ICER. The results of the immunocytochemistry studies support the idea that ICER is induced transiently in a stage-specific manner.

3.4. Inhibition of CREB-induced gene transcription by ICER

The functional properties of ICER in Sertoli cells were further examined in transient transcription assays. Primary Sertoli cells were transfected with a chloramphenicol acetyl transferase (CAT) reporter plasmid containing a region of the CREB promoter that includes the two CREs (CREBCAT). As was shown previously (Walker et al., 1995), transcription of the CAT reporter gene was induced approximately 5–6 fold by cotransfection of an expression vector encoding the catalytic subunit of protein kinase A (Fig. 4(A and C)). In contrast, cotransfection of the ICER expression vector reduced PKA-stimulated CAT activity to basal levels, whereas a vector expressing a control carboxyl-terminal deleted ICER mutant deficient in dimerization (ICER-mut) (Bodor et al., 1995) had little effect upon transcriptional activity. In transfection experiments employing the proenkephalin promoter, which also contains non-consensus CREs, ICER was also an effective repressor of PKA-mediated transcription (Fig. 4(B)). Together, these data show that in Sertoli cells ICER competes with CREB for occupancy of asymmetrical CREs in vivo and block transcription induction by the cAMP-dependent PKA pathway.

4. Discussion

The finding that the cAMP-responsive early repressor, ICER, is induced by FSH in Sertoli cells, binds to the CREs of the CREB promoter, and appears to down-regulate the expression of CREB may be an important point in understanding the stage-specific regulation of CREB expression in Sertoli cells (Waeber et al., 1991). With the exception of ICER, other isoforms of CREM, including the transcriptional activator CREMτ, are not expressed at high levels in Sertoli cells (Foulkes et al., 1992; Monaco et al., 1995); therefore, CREB is a promising candidate as a positive regulator

Fig. 3. ICER is induced in stage XII–XIV seminiferous tubules. A, Electrophoretic mobility shift assay of extracts from segments of microdissected rat seminiferous tubules. Partially purified bacterially expressed ICER (lane 1), CREB proteins (lane 2), no proteins (lane 3), and whole cell protein extracts from segments of seminiferous tubules representing stages VII–VIII, IX–X, XI–XII, XIII–XIV, I, II–III, and IV–VI were incubated with a ^{32}P-radiolabeled oligonucleotide probe containing the CREB CREs. The DNA-protein complexes were fractionated through a native polyacrylamide gel and identified by autoradiography. Complexes containing homodimers of CREB/CREM or ICER and CREB/ICER heterodimers are noted. The last three lanes (right) show that the addition of CREM/CREB-specific antiserum (αCREM) to the binding reaction containing extract of stage XIII–XIV tubule segments results in a retardation of the DNA (CREBCRE)-protein complex (Supershift, SS) not seen in control reactions without serum or with preimmune serum. A diagram of a rat seminiferous tubule is shown above the autoradiogram to provide a guide to the identification of the tubule segments identified by transillumination. B, immunostaining of adult rat testis with ICER-specific antiserum. Frozen sections of adult rat testis tissue were immunostained with ICER-specific antisera and a Cy3 fluorescent secondary anti-rabbit serum (panels A–C) or a biotin conjugated secondary antiserum (panel D): panel A, control preimmune serum staining at low magnification (50X). panel B, ICER specific antisera staining at low magnification. panel C, ICER-specific antisera staining (70X), panel D, ICER-specific antisera staining (70X); the brown staining is indicative of the immune avidin-biotin complex, nuclei have been stained blue with hemotoxilin. Staining of Sertoli cell nuclei (S) is identified by arrows. Zygotene spermatocytes (Z) pachytene spermatocytes (P), Diplotene spermatocytes (D), round spermatids (Sd) elongated spermatids (ES) and spermatozoa (Sz) are also indicated. Seminiferous tubules at various stages are shown in Roman numerals.

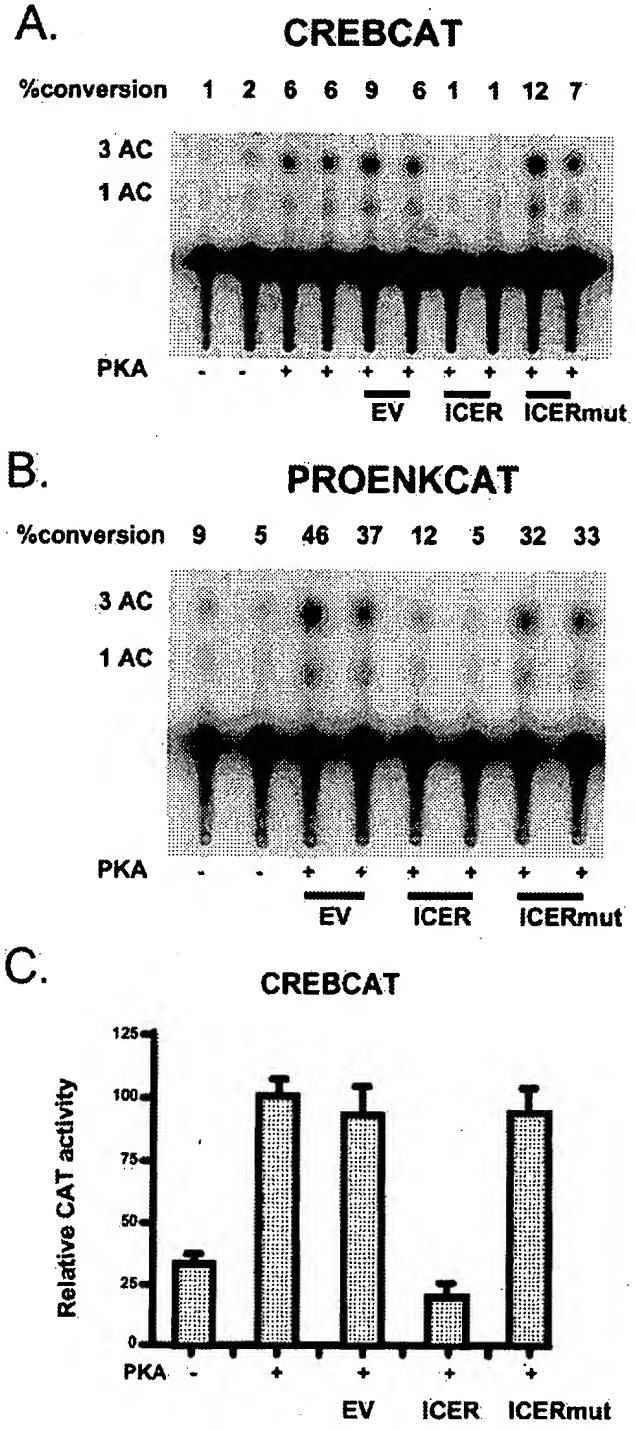

Fig. 4. ICER down-regulates the promoter of the CREB gene in Sertoli cells. Primary Sertoli cells were transfected with: A, the CREBCAT plasmid containing the CREB promoter region extending 278 bp 5′ to the translation start site linked to the CAT reporter gene (-278CREBCAT) (Walker et al., 1995), or B, PROENKCAT, the CREs in the context of the rat proenkephalin promoter (pENKAT-12) (Comb et al., 1986). Transfection assays were done with or without the PKA catalytic subunit (C_β) expression vector, pRSVCat-β (PKA) (Maurer, 1989) and empty pCMV5 expression vector (EV) pCMV5 containing the sequences encoding ICERIγ (ICER) or a mutant ICER in which the carboxyl-terminal leucine zipper is mutated (ICERmut) (Bodor et al., 1995). CAT activity is given as percent conversion of unacetylated to acetylated CAT products (3AC, 1AC). C, Summary of CREBCAT experiments. CAT activity is expressed relative to cells cotransfected with pRSVCat-β which was designated 100% activity. Results shown are from three separate transfections performed in duplicate. Standard errors of the mean are provided for each condition.

of cAMP-induced genes in Sertoli cells. CREB may be an important regulatory signal for a number of cAMP-regulated genes that have been studied in Sertoli cells, including the proto-oncogenes c-fos (Hall et al., 1988), junB (Smith et al., 1989), and α-inhibin (Najmabadi et al., 1993), as well as proenkephalin (Yoshikawa and Azawa, 1988) and androgen-binding protein (Joseph et al., 1988). The cAMP-regulated control of CREB gene expression, therefore, may be critical for the regulation of several genes required for the maturation of germ cells.

Our studies suggest that the expression of CREB and ICER in Sertoli cells in vivo may be cyclically regulated via the production of cAMP induced by FSH that occurs during cell association stages XII-V (Kangasniemi et al., 1990). The ICER gene appears to rapidly respond to increased cAMP levels inasmuch as the highest levels of ICER are detected by immunocytochemistry during stages XII–XIV. The levels of ICER then return to basal levels after stage I. In contrast to the temporal pattern of the induction of ICER, induction of the CREB gene by cAMP is delayed until stages II–V. Because ICER is a candidate repressor of the CREB gene, it may be an important factor responsible for the delayed induction of the CREB gene by FSH and cAMP in Sertoli cells. Together, the two regulators, CREB and ICER, may be responsible for limiting the expression of cAMP-inducible genes in Sertoli cells to specific stages of germ cell development.

As depicted in Fig. 5, ICER interrupts a hypothetical positive auto-feedback loop responsible for the high levels of CREB detected in Sertoli cells at specific stages of spermatogenesis. In response to stimulation by FSH, cAMP levels increase, resulting in the activation of the PKA catalytic subunit and the phosphorylation of CREB. The activation of CREB results in the stimulation of the CREB promoter and thereby increases levels of CREB mRNA and protein. At the same time, the ICER promoter is stimulated by CREB, causing ICER levels to increase, which eventually feed back to suppress the ICER gene promoter. The delay in the induction of CREB by FSH-mediated cAMP formation may be due to the induction of the ICER repressor which transiently occupies the cAMP-response elements present in the promoter of the CREB gene until ICER levels eventually fall (Molina et al., 1993).

The gene encoding the FSH receptor is also down-regulated by ICER in Sertoli cells in response to stimulation by FSH (Monaco et al., 1995). Therefore, ICER

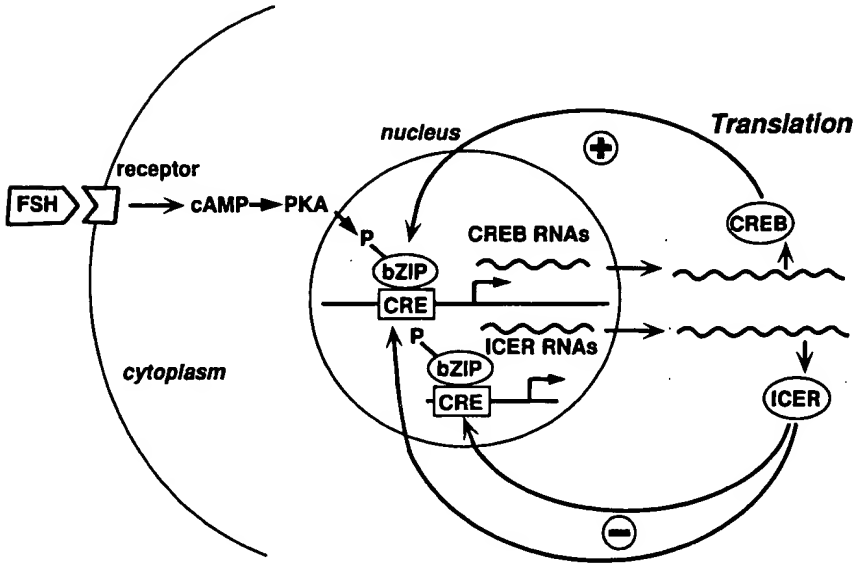

Fig. 5. CREB gene expression is cyclically stimulated and repressed during the spermatogenic cycle. A model of CREB gene expression during spermatogenesis shows that at the initiation of a new round of germ cell development FSH induces stimulation of the production of cAMP and activates PKA to phosphorylate CREB. When phosphorylated, CREB bound to the CREB promoter stimulates CREB transcription, producing more CREB. Meanwhile, CREB activates ICER transcription, causing the production of ICER repressor. Above some threshold level, ICER causes the down regulation of cAMP-induced genes including the CREB gene. Within 6–12 h ICER acts to repress transcription from the ICER promoter within the CREM gene. ICER then slowly degrades allowing cAMP promoters to be reset to basal activities until cAMP levels rise again.

may down-regulate the expression of the CREB gene by two independent but cooperative mechanisms: by eliminating the ability of FSH to raise cellular cAMP levels or by a direct blockade of the activation of the CREB promoter. This level of coordinate control of CREB gene expression suggests that CREB may be an important regulatory factor in Sertoli cells during the spermatogenic cycle.

It is notable that in vitro, ICER-binding activity in Sertoli cells is induced by 6–12 h after FSH stimulation and that binding activity declines by 24 h and reappears at 36–48 h. FSH-induced ICER levels in Sertoli cells as detected by Western immunoblot, however, remain elevated for up to 36 h (Monaco et al., 1995). In the seminiferous tubule, the time from the beginning of the induction of ICER (Stage XII) to the time of deinduction (Stage XIV) is approximately 48 h, based on the accuracy by which we can estimate the expression of ICER in the segments of seminiferous tubules. Therefore, it would appear that the interval for the induction of ICER in the seminiferous tubule in vivo, approximates that of Sertoli cells in culture.

The long half-life of ICER does not reconcile with the reported findings that FSH receptor mRNA levels are repressed for 2–4 h after FSH-stimulated production of ICER but rebound to pretreatment levels after 8–24 h of FSH treatment (Monaco et al., 1995). A similar pattern of regulation was reported earlier in Sertoli cells for the induction of the expression of the CREB gene in response to FSH (Walker et al., 1995). It would appear, therefore, that although ICER levels may remain elevated 12–24 h after stimulation, the major repressor activity of ICER on some genes is short-lived. Perhaps the action of ICER is to promptly inhibit CRE-mediated transcription of cAMP responsive target genes including the CREB and FSH receptor genes, and then to allow basal levels of transcription to be reinitiated. The reaccumulation of FSH receptors and CREB then allows the system to be reset and to be triggered by the cAMP-mediated phosphorylation of CREB again.

Acknowledgements

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CREB Phosphorylation Promotes Nerve Cell Survival

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Abstract: The cyclic AMP-responsive element binding protein (CREB) is a posttranslationally activated transcription factor that has been implicated in numerous brain functions including cell survival. In this study we investigated whether CREB overexpression using transient transfection of a pAAV/CMV-CREB plasmid altered neuronal cells' susceptibility to apoptosis. We found that elevated CREB protein inhibited apoptosis induced by okadaic acid. At least part of this effect is critically dependent on prolonged Ser^{133} phosphorylation, as a directed mutation at this site decreased CREB-induced protection. These results suggest that CREB is a survival factor for neuronal cells and that treatments aimed at augmenting CREB phosphorylation in the brain may be neuroprotective. **Key Words:** Cyclic AMP-responsive element binding protein—Neuroprotection—Survival—Okadaic acid—Apoptosis.
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The cyclic AMP (cAMP)-responsive element binding protein (CREB) is a 43-kDa protein that has been implicated in the transcriptional control of many genes, in particular those that are rapidly induced by elevation in cytoplasmic cAMP or calcium. Although CREB is normally expressed at high levels, its activity at the Ca^{2+}/cAMP-responsive element (CRE) is dependent on phosphorylation at Ser^{133} by a number of protein kinases (Yamamoto et al., 1988; Sheng et al., 1991; Enslen et al., 1994; Ginty et al., 1994; Matthews et al., 1994; Xing et al., 1996). The mechanism by which Ser^{133} phosphorylation activates CREB remains unclear; however, it has been suggested that this modification induces a conformational change that transforms CREB from an inactive to an active configuration (Gonzalez et al., 1991; Brindle et al., 1993). Alternatively, phosphorylated CREB (pCREB) may enhance transcription via recruitment of coactivators such as CREB binding protein (Chrivia et al., 1993; Arias et al., 1994; Kwok et al., 1994).

In this active form, CREB regulates many aspects of neuronal functioning, including excitation of nerve cells (Moore et al., 1996), circadian rhythms (Ginty et al., 1993), CNS development (Imaki et al., 1994), pituitary proliferation (Struthers et al., 1991), and long-term memory formation (Silva et al., 1998). Recently, we found that following hypoxic-ischemic brain injury, increased levels of pCREB were evident in the damage-resistant neurons of the dentate gyrus and neocortex but not in the vulnerable CA1 neurons that undergo delayed apoptosis. This association with resistant cell populations suggested that the activation of CREB might have a protective role in the CNS (Walton et al., 1996). To determine whether CREB (expression and/or phosphorylation) is indeed a survival factor for neurons, we expressed this protein in cultured neuron-like cells and measured their susceptibility to okadaic acid (OA)-induced apoptosis.

MATERIALS AND METHODS

Cell culture

PC12 cells (generous gift from Eric Shooter) were maintained at 7.5% CO_2/37°C in Dulbecco's modified Eagle's medium supplemented with 6% horse serum, 6% fetal calf serum, L-glutamine (2 m*M*), penicillin (100 U/ml), and streptomycin (100 μg/ml; all from GibcoBRL). All experiments were performed on undifferentiated cells plated at a density of 60,000 cells/cm^2 on rat tail collagen (5 μg/cm^2; Sigma)-coated culture plates. The Neuro2A (ATCC no. CCL-131) mouse neuroblastoma cells were maintained at 5% CO_2/37°C in RPMI 1640 medium (GibcoBRL) supplemented with 10% fetal calf serum, L-glutamine (2 m*M*), penicillin (100 U/ml), streptomycin (100 μg/ml), and sodium pyruvate. For transfection and cytotoxic assays, cells were plated at 30,000 cells/cm^2. To differentiate Neuro2A following transfection, the cells were grown for 5 days in the presence of retinoic acid (1×10^{-5} *M*; Sigma).

Transient transfections

Cells were transfected with plasmids containing either a *CREB* (pAAV/CMV-CREB) or a *LacZ* (pAAV/CMV-lacZ)

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Abbreviations used: AAV, adeno-associated virus; BDNF, brain-derived neurotrophic factor; cAMP, cyclic AMP; CMV, cytomegalovirus; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; EMSA, electrophoretic mobility shift assay; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OA, okadaic acid; pCREB, phosphorylated CREB.

cDNA transcription cassette under the control of a cytomegalovirus (CMV) promoter flanked by adeno-associated virus (AAV)-inserted terminal repeats. For mutational analysis, cells were transfected with a CREB mutant [*CREB(S133A)*] plasmid in which the Ser^{133} residue was replaced with an alanine. This was carried out as follows: Based on the sequence of CREB cDNA [(383) T TCA AGG AGG CCT TCC TAC AGG (404)] cloned in the pGEM-3Zf(−) vector, the following oligonucleotide was synthesized: T TCA AGG AGG CCT GCC TAC AGG. With use of this primer and another one from the pGEM-3Zf(−) plasmid, a 749-bp fragment of the CREB cDNA with the substitution of T for G in the 396 position (Ser^{133}–Ala) was amplified. This fragment was cut with *Stu*I and *Hin*dIII enzymes and cloned into the pGEM-3Zf(−) vector (CREB) in which the same unmodified region had been removed by a two-step process.

PC12 cells were transfected according to the Dosper transfection protocol (Boehringer-Mannheim). To assess the transfection efficiency, the percentage of immunopositive cells was determined from five fields/well (n = 9 wells/treatment). Neuro-2A cells were transfected with the *CREB, CREB(S133A), C/ebp-α* (generously provided by Steven McKnight) (Friedman et al., 1989), *LacZ,* and *pCRE-luc* (cat. no. 219076; Stratagene) plasmids using Fugene 6 transfection reagent (Boehringer-Mannheim), which we have found to be more efficient than Dosper on this cell line (unpublished observations). For studying cell susceptibility, the cells were transfected the day before a 24-h exposure to the toxin. The extent of cell death was then evaluated using reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma), Hoechst no. 33258 (bisbenzimide; Sigma) staining, and lactate dehydrogenase (LDH) release (MacGibbon et al., 1997).

Immunocytochemistry

Immunocytochemical staining (previously described by Walton et al., 1998*b*) was carried out to check protein expression following transfection. We used primary antibodies against rat CREB, rat pCREB [rabbit polyclonal antisera; cat. nos. 06-504 (1:4,000) and 06-519 (1:500), respectively; Upstate Biotechnology], β-galactosidase [mouse monoclonal; cat. no. 19929-017 (1:250); GibcoBRL], and luciferase [rabbit polyclonal; cat. no. E4191 (1:5,000); Promega].

Immunofluorescence/propidium iodide staining was performed to evaluate the nuclear integrity of CREB-transfected cells. Cells were transfected with either the *CREB* or *LacZ* plasmid for 24 h and then treated with either vehicle or OA (200 n*M;* Research Biochemicals International) for 48 h. The primary antibodies were applied as described above. After extensive washing, the CREB-positive signals were developed by a 2-h incubation with a fluorescent secondary anti-rabbit antibody conjugated to Cy2 (1:250; Sigma). The β-galactosidase-positive cells were identified by 2-h incubation with an anti-mouse biotinylated secondary antibody (1:250; Sigma) followed by a 2-h incubation with ExtrAvidin conjugated to fluorescein isothiocyanate (1:250; Sigma). Following extensive washing, the cells were incubated with propidium iodide (50 ng/ml; Sigma) for 1 h. The nuclear morphology of the transfected cells was examined using a fluorescent microscope at ×200 magnification. The percentages of CREB-, LacZ-, and nontransfected cells in the wells that contained either fragmented or condensed nuclei were determined from five fields/well (n = 20–24 wells/treatment).

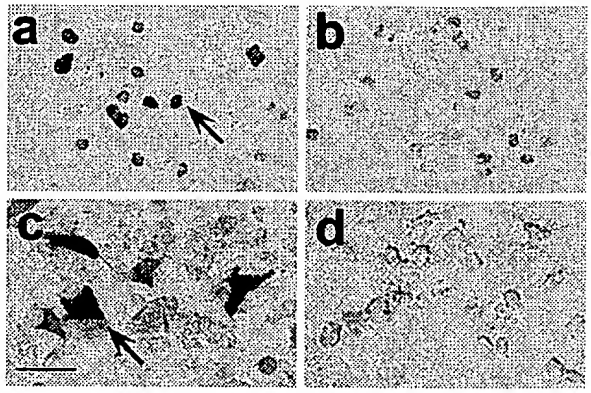

FIG. 1. Photomicrographs of CREB (a and b) and β-galactosidase (c and d) immunoreactivity in cells transfected with the *CREB* (a and d) and *LacZ* (b and c) plasmids. Arrows show immunopositive cells. Bar = 40 μm.

Electrophoretic mobility shift assay (EMSA)

Cells were transfected with either the *CREB, CREB(S133A),* or *LacZ* plasmid for 24 h (30×10^6 cells/treatment). The EMSA was then carried out as described previously by Walton et al. (1998*a*). A double-stranded oligonucleotide containing the consensus sequence for CRE (Santa Cruz Biotechnology, no. 2504; 5′ AGA GAT TGC CTG ACG TCA GAG AGC TAG 3′) was 5′ end-labeled with [γ-^{32}P]ATP (3,000 Ci/mmol) with T4 polynucleotide kinase (Boehringer-Mannheim). For competition studies, unlabeled CRE and Ets oligonucleotides (Santa Cruz Biotechnology, no. 2549; 5′ GGG CTG CTT GAG GAA GTA TAA GAA T 3′) in 10- to 100-fold excess were included in the reaction mixture. For supershift studies, extracts were incubated (20 min) with the anti-rat pCREB antiserum and normal rabbit serum at 1:10 dilution 15 min after the addition of labeled probe.

RESULTS

Characteristics of CREB transfection

A considerable increase in CREB immunoreactivity (24–72 h) was found in the nucleus of cells transfected (~8% transfection efficiency) with the pAAV/CMV-CREB plasmid but not with the pAAV/CMV-LacZ plasmid (Fig. 1). No CREB immunoreactivity was observed in the nucleus of cells transfected with the marker gene *LacZ,* indicating that CREB expression is not a byproduct of the transfection procedure, as has been suggested for JNK (c-Jun N-terminal kinase)-mediated effects (Bruening et al., 1998). Immunofluorescent double-labeling studies showed that 100% of the CREB-overexpressing cells were phosphorylated at Ser^{133} (<5% of normal PC12 cells show CREB phosphorylation; results not shown), an observation that was probably the direct result of a constitutively active CREB kinase within these cells. However, despite CREB protein overexpression and phosphorylation, no noticeable morphological alterations were evident in CREB-transfected PC12 cells.

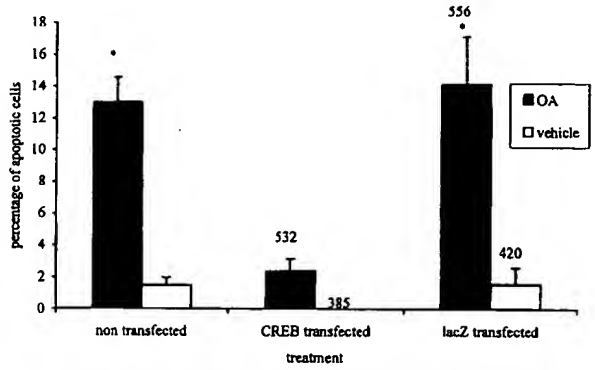

FIG. 2. Graph showing the percentage of CREB- and β-galactosidase (LacZ)-overexpressing cells that exhibit apoptotic morphology after 48-h exposure to OA (200 n*M*) (+24-h transfection). SE bars are shown about the mean. Statistical significance was evaluated by ANOVA (OA: $^*p < 0.001$, significant; vehicle: $p = 0.730$, not significant). For OA, the Tukey multiple comparison test showed that the percentage of apoptotic cells transfected with the *CREB* plasmid was significantly different from that observed in the *LacZ*-transfected cells. Numbers above columns indicate the numbers of transfected cells counted.

Susceptibility of CREB-transfected cells toward OA-induced cell death

Immunofluorescence/propidium iodide staining was carried out to determine whether CREB-overexpressing PC12 cells were less susceptible toward apoptosis than cells transfected with the marker gene *LacZ*. To induce apoptosis in these cells, we used exposure to OA, a polyether fatty acid that potently inhibits protein phosphatases 1 and 2A. Treatment with this marine shellfish toxin has been associated with apoptotic death in a variety of cell types, including PC12 cells (Walton et al., 1998*b*; Woodgate et al., 1999). A 48-h exposure to OA (200 n*M*) produced the ultrastructural features of apoptosis, including membrane blebbing, nuclear (and DNA) fragmentation, and chromatin condensation. Approximately 13% of the total (transfected + nontransfected) cells contained either condensed or fragmented nuclei. A similar percentage of the β-galactosidase (LacZ-transfected)-positive cells exhibited apoptotic morphology. In contrast, these characteristics were apparent in only 2% of the CREB-transfected cells (Fig. 2).

To confirm the protective effect of CREB, we used two other markers of cell viability: MTT and LDH release. A significant decrease in MTT reduction combined with an increase in medium LDH (indicative of secondary necrosis) was observed following 24- to 48-h exposure to OA. However, as these assays rely on the combined properties of a number of cells and the transfection efficiency is relatively low in PC12 cells, it was necessary to turn our attention to the Neuro2A mouse neuroblastoma cells, which are easier to transfect (20–30% efficiency) using another transfection reagent, Fugene 6. Differentiation of these cells using retinoic acid (10^{-5} *M*) produced a neuron-like morphology with considerable neurite extension (Fig. 3). This cell line also showed a similar pattern of OA toxicity to that found in PC12 cells.

Confirming the PC12 cell results, overexpression of CREB protein in Neuro2A cells increased their resistance to OA-induced toxicity. Differentiated Neuro2A cells transfected with the *CREB* plasmid showed a significantly larger MTT absorbance following OA than that found in similar *LacZ*-transfected cells. A comparable difference in MTT reduction was also found in undifferentiated Neuro2A cells (Fig. 4A), suggesting that the differentiation state had no influence on the protection conveyed by CREB overexpression. Accordingly, all further characterization studies were carried out in undifferentiated cells. To ensure that this effect was due to CREB-induced neuroprotection and not to a nonspecific *LacZ* toxicity, we used ectopically expressed C/EBP-α as an additional control. The fact that there was no significant difference in MTT absorbance between the LacZ- and C/EBP-α-transfected cells in either the vehicle- or the OA-treated group rules out the possibility that β-galactosidase overexpression was itself toxic (Fig. 4A).

We also investigated whether elevated CREB levels resulted in an overall protective effect as measured by the number of cells remaining in the culture well. Following OA treatment, some of the dead or dying cells displace from the plastic of the plate and tend to be removed during processing, resulting in a decrease in

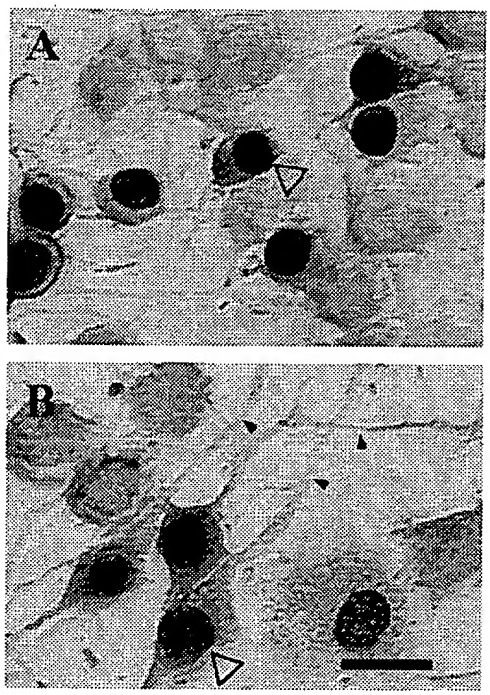

FIG. 3. Photomicrographs showing CREB immunoreactivity in undifferentiated (**A**) and differentiated (**B**) Neuro2A cells transfected with the *CREB* plasmid (open arrowheads). Note the long process extensions in the retinoic acid-differentiated cells (filled arrowheads). Bar = 26 μm.

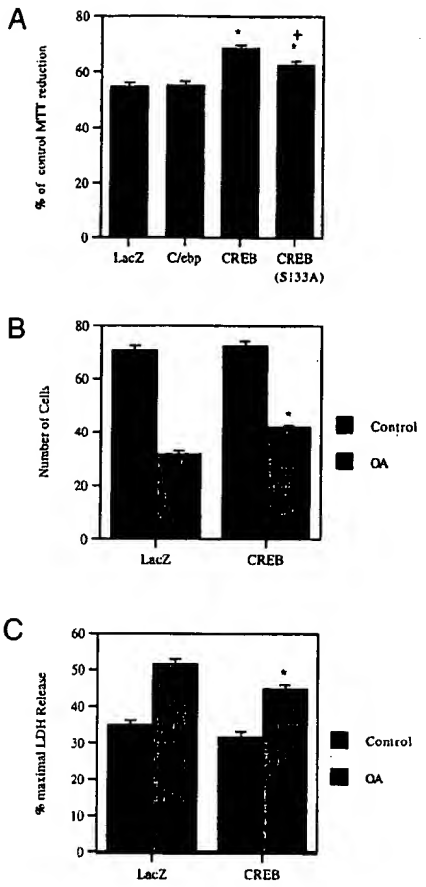

FIG. 4. Graphs showing MTT reduction (**A**), total cell counts (**B**), and LDH release (**C**) in transfected cells 24 h following OA treatment. SE bars are shown about the mean. Statistical significance was evaluated by ANOVA [considered significant; (A) $F_{3,69} = 29.062$; (B) $F_{3,53} = 222.01$; (C) $F_{3,34} = 44.087$] followed by a Tukey post hoc multiple comparison test, $^{*,+}p < 0.05$. * represents statistical significance between CREB- and LacZ-transfected cells in the presence of OA; + represents statistical significance between CREB and CREB(S133A).

absolute cell number. Using Hoechst 33258 staining as a marker of intact nuclei, we found that the number of adherent cells in the CREB-transfected group was ~30% greater than in the LacZ-transfected group (Fig. 4B). This decrease in OA toxicity not only supports the results obtained using the MTT assay, but its magnitude matched the percentage of cells successfully transfected with the *CREB* plasmid. To determine whether up-regulated CREB expression may also have an effect on the secondary necrosis that is characteristic of OA toxicity, we used LDH release as a measure of cell lysis. The increase in LDH release of OA-treated Neuro2A cells was significantly less in the cells ectopically expressing CREB than LacZ, again supporting a role for this protein in cellular protection (Fig. 4C).

Phosphorylation of CREB at Ser[133]

The fact that the overexpressed CREB protein in both PC12 and Neuro2A cells appears to be phosphorylated at Ser[133] prompted us to determine whether it is this modification that underlies its protective effect, as suggested by a previous in vivo study (Walton et al., 1996). To achieve this, we transfected Neuro2A cells with a plasmid [*CREB(S133A)*] identical to *CREB* except for a substitution so that alanine was produced instead of serine at amino acid 133. Such a mutation prevents phosphorylation at this site and has been shown to reduce CREB activity in a number of model systems. This observation was supported by EMSA where the levels of CRE binding were substantially increased in the *CREB* but not in the mutant-transfected cells (Fig. 5). Furthermore, cotransfection with a plasmid containing the luciferase reporter gene driven by the CRE promoter (*pCRE-Luc* plasmid) indicates that the Ser[133] mutation reduces not only CREB's DNA binding activity but also its transcriptional activity (Fig. 5). As expected, this modification also affected CREB functionally, with a significantly lower MTT score in the mutant transfected cells than in the wild type (Fig. 4A). Although the serine–alanine substitution in the CREB protein reduced its ability to promote cell survival, it was unable to eliminate it totally. In fact, cells were still significantly more resistant to OA toxicity than those expressing the marker gene *LacZ* (Fig. 4A).

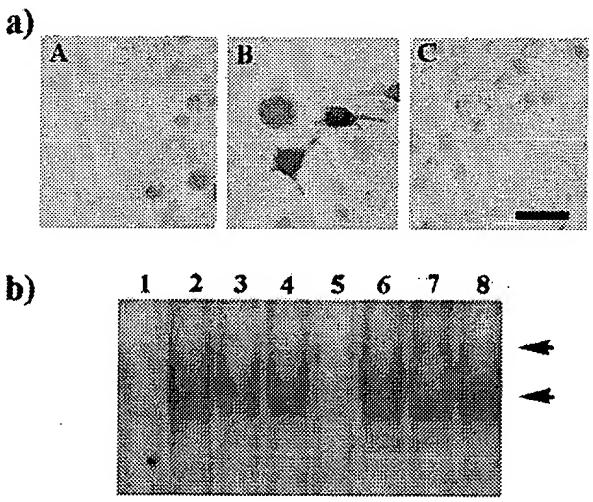

FIG. 5. Characterization of CRE activity and binding in cells transfected with the *LacZ*, *CREB*, and *CREB(S133A)* plasmids. **a:** Luciferase immunoreactivity on cells cotransfected with *pCRE-luc* and *LacZ* (**A**), *CREB* (**B**), or *CREB(S133A)* (**C**). Bar = 35 μm. **b:** Electrophoretic mobility shift CRE-binding assay on protein extracted from cells transfected with the *LacZ* (lane 1), *CREB* (lane 2), or *CREB(S133A)* (lane 3) plasmids. The binding in the CREB-transfected cells was displaced by 10× (lane 4) and 100× (lane 5) excess cold CRE but was unaffected by the nonspecific Ets probe (lane 6). The addition of the pCREB antibody (lane 7) to the reaction mixture produced a supershifted band, whereas normal rabbit serum (lane 8) had no effect on the CRE binding. Bottom arrow shows CRE-CREB retarded band. Top arrow shows pCREB supershifted band.

DISCUSSION

In addition to an active death process leading to apoptosis, it is equally likely that resistance to a particular cellular insult is the result of an intrinsic survival mechanism involving a series of transcriptional events. The presence and/or activity of such a pathway in any particular cell type may explain their varying sensitivities to an insult. A potentially important component in this survival cascade is the phosphorylation of CREB. Not only is this protein activated in resistant cell populations following a hypoxic–ischemic brain injury in vivo (Walton et al., 1996; Hu et al., 1999; Tanaka et al., 1999), but the current study shows that pCREB-overexpressing cells were less susceptible to OA-induced apoptosis. In support of this antiapoptotic property, human melanomas and T cells expressing a dominant negative form of CREB showed decreased cellular resistance to a number of apoptosis-inducing stimuli including radiation and thapsigargin (Barton et al., 1996; Yang et al., 1996; Jean et al., 1998). Moreover, elevated cAMP level, an event known to activate CREB, is sufficient to promote the survival of spinal motor neurons in vitro (Hanson et al., 1998).

The link between the levels of pCREB and programmed cell survival raises some important questions about the identity of up- and downstream components in this signaling pathway. Interestingly, it has been shown that pCREB not only mediates the cellular response to nerve growth factor and brain-derived neurotrophic factor (BDNF) via the activity of various kinases (Bonni et al., 1995; Finkbeiner et al., 1997) but also is able to directly regulate BDNF transcription (Tao et al., 1997). These findings combined with the fact that BDNF colocalizes with pCREB in resistant cell populations in vivo (Walton et al., 1999) suggest that a interrelationship with neurotrophins may be important to CREB's protective effect. Another cascade that may initiate CREB-induced protection against apoptosis in physiological conditions is the signal pathway from phosphoinositide 3-kinase to the serine/threonine kinase Akt/PKB (Dudek et al., 1997). Recently, Du and Montminy (1998) showed that Akt/PKB promotes cell survival at least in part by stimulating gene expression in a pCREB-dependent manner. This pathway may be involved in the neuroprotective effects of BDNF as phosphoinositide 3-kinase activity is important to its ability to promote survival (Shimoke et al., 1997). Although the identity of downstream target genes is not clear, pCREB can trigger induction of the antiapoptotic gene bcl-2, which contains a CRE site in its promoter (Wilson et al., 1996). Indeed, Wilson et al. (1996) found that pCREB-mediated bcl-2 expression is centrally involved in the rescue of immature B cells from calcium-dependent apoptosis.

The persistent nature (>24 h) of the CREB overexpression in transfected cells may be seen as nonphysiological. However, this model system could be viewed as underlining the importance of prolonged CREB phosphorylation in neuroprotection (Bito et al., 1996; Liu and Graybiel, 1996), as distinct from that produced by other stimuli where the response is typically rapid and short lived (Bonni et al., 1995). Indeed, hypoxia in vivo and in vitro has been shown to produce a biphasic CREB phosphorylation that persists for at least 24 h (Walton et al., 1996; Beiter-Johnson and Millhorn, 1998). This hypoxia-induced CREB phosphorylation in PC12 cells is likely to underlie a number of adaptive cellular responses to a reduction in oxygen levels, some of which may include protection against death (Beiter-Johnson and Millhorn, 1998).

It is interesting to note that in addition to inducing cell death, OA leads to the prolonged phosphorylation of constitutively expressed CREB by the inhibition of protein phosphatases (Woodgate et al., 1999). However, it remains to be determined whether this event is directly associated with OA-induced apoptosis rather than with some other unrelated mechanism. Even so, this discrepancy raises some important issues related to the possibility that both cell death and cell survival programs can be activated by the same insult. In PC12 cells, OA does not induce a homogeneous pattern of cell death but instead results in distinct populations that appear less susceptible than others (Walton et al., 1998; Woodgate et al., 1999). Furthermore, OA leads to ATF-2 phosphorylation, which we believe mediates its neurotoxic effects (Walton et al., 1998*b*). The presence of activated CREB in a particular cell may delay the onset of death by countering these proapoptotic effects and therefore be responsible for the variations in injury resistance within a cell population.

It appears that a significant proportion of CREB-induced protection against apoptosis is mediated by the phosphorylation of CREB at Ser^{133} and subsequent CRE activity. However, the fact that the mutation of Ser^{133} reduced but did not eliminate this protective effect suggests that an alternative mechanism is operating parallel to that of pCREB/CRE activation. One possibility is that the phosphorylation of CREB at other serine residues is important to its function in cell survival. Indeed, Fiol et al. (1994) showed that secondary phosphorylation of CREB at Ser^{129} is required for the cAMP-mediated control of gene expression. Alternatively, CREB by binding to a consensus sequence such as AP-1 in a phosphorylation-independent manner may inhibit the progression of apoptosis. CREB in its nonphosphorylated form can suppress AP-1 activity by competing with Jun protein for the AP-1 site (which differs from the CRE element by only one nucleotide) on target genes (Lamph et al., 1990; Masquilier and Sassone-Corsi, 1992).

The observation that CREB overexpression (and phosphorylation) protects cells against toxin-induced apoptosis in vitro and is phosphorylated in damage-resistant neurons in vivo (Walton et al., 1996) suggests that endogenous CREB activation is a potent survival signal in times of cellular stress. The CREB protein and the CRE site are therefore potential targets for both existing and future neuroprotective agents. Moreover, CREB is crucial to the establishment of long-term memory (Silva et

al., 1998), and any molecular or pharmacological manipulation is likely to influence cognition as well as neuroprotection. The idea that an increase in CREB activity may enhance not only cell survival but also long-term memory is an intriguing possibility that may be potentially useful in the treatment of neurodegenerative diseases such as Alzheimer's disease, in which memory impairments are combined with death of neurons.

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Impaired Cyclic AMP-Dependent Phosphorylation Renders CREB a Repressor of C/EBP-Induced Transcription of the Somatostatin Gene in an Insulinoma Cell Line

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Transcription factor CREB regulates cyclic AMP (cAMP)-dependent gene expression by binding to and activating transcription from cAMP response elements (CREs) in the promoters of target genes. The transcriptional transactivation functions of CREB are activated by its phosphorylation by cAMP-dependent protein kinase A (PKA). In studies of many different phenotypically distinct cells, the CRE of the somatostatin gene promoter is a prototype of a highly cAMP-responsive element regulated by CREB. We now report on a somatostatin-producing rat insulinoma cell line, RIN-1027-B2, in which transcription from the somatostatin gene promoter is paradoxically repressed by CREB. We find that CREB fails to transactivate a CRE-containing somatostatin-chloramphenicol acetyltransferase reporter even when coexpressed with the catalytic subunit of PKA. CAAT box/enhancer-binding protein β (C/EBPβ) and C/EBP-related activating transcription factor bind to the CRE in the promoter of the somatostatin gene and transactivate transcription. CREB binds competitively with C/EBPβ to the somatostatin CRE in vitro and represses C/EBPβ-induced transcription of the CRE-containing somatostatin-chloramphenicol acetyltransferase reporter. The lack of CREB-mediated transcriptional stimulation is due to the presence of a heat-stable inhibitor of PKA that prevents activation of PKA and subsequent CREB phosphorylation in the nucleus. These findings indicate that dephosphorylated CREB is a negative regulator of C/EBP-activated transcription of the somatostatin gene promoter in RIN-1027-B2 cells.

Adaptive changes in the functional state of cells in response to various stimuli in the environment involve the activation of cellular second messenger pathways that cause changes in the transcription rates of target genes. Many extracellular signals are transmitted to the nucleus by pathways that selectively activate protein kinases and the resultant phosphorylation of transcription factors that in turn regulates the expression of specific genes (18). The cyclic AMP (cAMP)-dependent signal transduction pathway mediates transcriptional responses of many genes. The catalytic subunits of protein kinase A (PKA) released from the cytoplasmic inhibitory regulatory subunits by the actions of cAMP translocate to the nucleus, where they phosphorylate transcription factors such as CREB at specific serine residues, resulting in the activation of gene transcription (18, 27, 53).

CREB binds specifically to cAMP response elements (CREs) typified by the consensus palindromic sequence TGACGTCA, present in the promoters of many genes, including the gene encoding the polypeptide hormone somatostatin, in which transcription rates are strongly regulated by cAMP (10, 20, 28, 35, 51). In particular, the CRE in the promoter of the somatostatin gene is highly responsive to cAMP stimulation and as such has been used extensively as a prototype CRE for the study of cAMP-dependent mechanisms of transcriptional regulation (9, 13, 19, 20, 28). These studies, however, have been carried out with heterologous cells that do not express the endogenous somatostatin gene, using transcription reporter plasmids consisting of the somatostatin CRE driving heterologous promoters.

The expression of the somatostatin gene is restricted to neurons, thyroid C cells, D cells of the digestive tract, and D cells of the pancreatic islets of Langerhans (38). In the somatostatin-producing insulinoma cell line RIN-1027-B2 (B2 cells) (34), the somatostatin gene is regulated by several cell-specific elements that exert positive or negative control on gene transcription (36, 54). Earlier studies using B2 cells and various other cell lines and transfection assays clearly demonstrate a pivotal role of the CRE in the regulation of cell-specific expression of the somatostatin gene (2, 22, 36, 54). Despite the demonstration of an essential role for the CRE of the somatostatin gene in mediating cAMP-dependent stimulation of gene transcription in different cell types, cAMP does not stimulate transcription in B2 cells (32, 36, 54).

To analyze this apparent paradox, we examined the cellular mechanisms that might be responsible for the observed lack of response to cAMP. We find that CAAT box/enhancer-binding protein (C/EBP)-like CRE-binding proteins potently activate transcription of the somatostatin gene. CREB, one of the nuclear proteins bound by the CRE in B2 cells, is not phosphorylated by PKA because of the presence of a heat-stable kinase inhibitor. As a consequence, dephosphorylated CREB (dephospho-CREB) represses transcription of the somatostatin gene by competing with C/EBP proteins for binding to the CRE. We identify C/EBP-related activating transcription factor (C/ATF) (56) and C/EBPβ (7, 60) as two of the major nuclear proteins that activate transcription of the somatostatin gene in a cAMP-independent manner.

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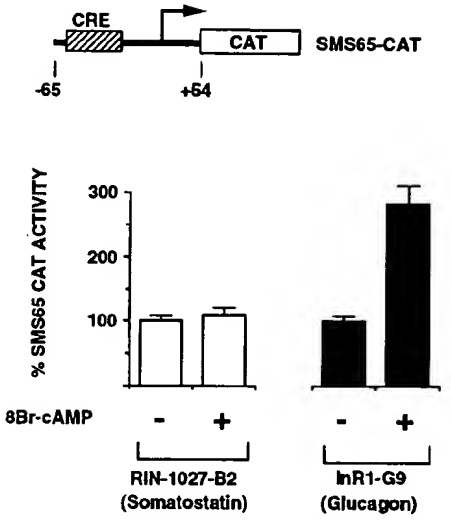


FIG. 1. Relative CAT activities obtained following transient transfections of somatostatin gene 5′ deletion plasmid SMS65-CAT into islet cell-derived somatostatin-producing B2 cells or glucagon-producing InR1-G9 cells cultured in the absence (−) or presence (+) of 1 mM 8-Br-cAMP for 12 h prior to harvesting. Values are expressed as percentages of the CAT activities elicited by SMS65-CAT in the absence of 8-Br-cAMP stimulation. A schematic representation of the SMS65-CAT reporter plasmid is depicted at the top of the figure.

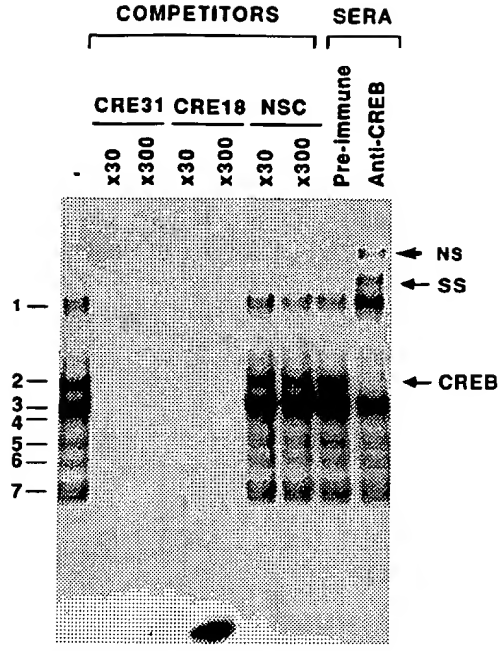


FIG. 2. EMSA of nuclear proteins from B2 cells bound to the somatostatin gene CRE. Competitions were with a 30- (×30) or 300-fold (×300) molar excess of unlabeled full-length somatostatin CRE oligonucleotide (CRE31), a truncated version spanning the core CRE and 5 flanking nucleotides at either side (CRE18), or a nonspecific competitor (NSC) oligonucleotide of unrelated sequence. A control (no competitor) (−) is shown. Antiserum supershift experiments were carried out by the addition to the binding reaction mixture of either anti-CREB specific or preimmune sera. Complex 2 supershifted (SS) by the CREB antiserum and a nonspecific band (NS) are shown. The positions of the complexes are shown to the left of the gel.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes were purchased from New England Biolabs (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Radioactive compounds were obtained from Du Pont-New England Nuclear (Boston, Mass.). Nucleotides were purchased from Pharmacia-LKB (Piscataway, N.J.). Tissue culture media and reagents were obtained from Gibco-BRL (Grand Island, N.Y.). All other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cell lines. Rat islet somatostatin-producing B2 (34), hamster islet glucagon-producing InR1-G9 (49), mouse NIH 3T3 (ATCC CRL1658), and human choriocarcinoma JEG-3 (ATCC HTB36) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Antisera. CREB, C/EBPβ, C/ATF, and PKA$_c$ (catalytic subunit of PKA) antisera are described elsewhere (15, 39, 56, 58). C/EBPα antiserum was raised by immunizing rabbits with a protein consisting of glutathione *S*-transferase (GST) fused to the amino-terminal portion of C/EBPα, excluding the basic region-leucine zipper (bZip) domain. C/EBPδ antiserum was a gift from S. L. McKnight (Tularik, Inc). Affinity-purified phosphorylated CREB (phospho-CREB) antibody was a gift from M. E. Greenberg (Harvard Medical School). Western immunoblots were carried out with a chemiluminescent detection system (ECL; Amersham), using an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody.

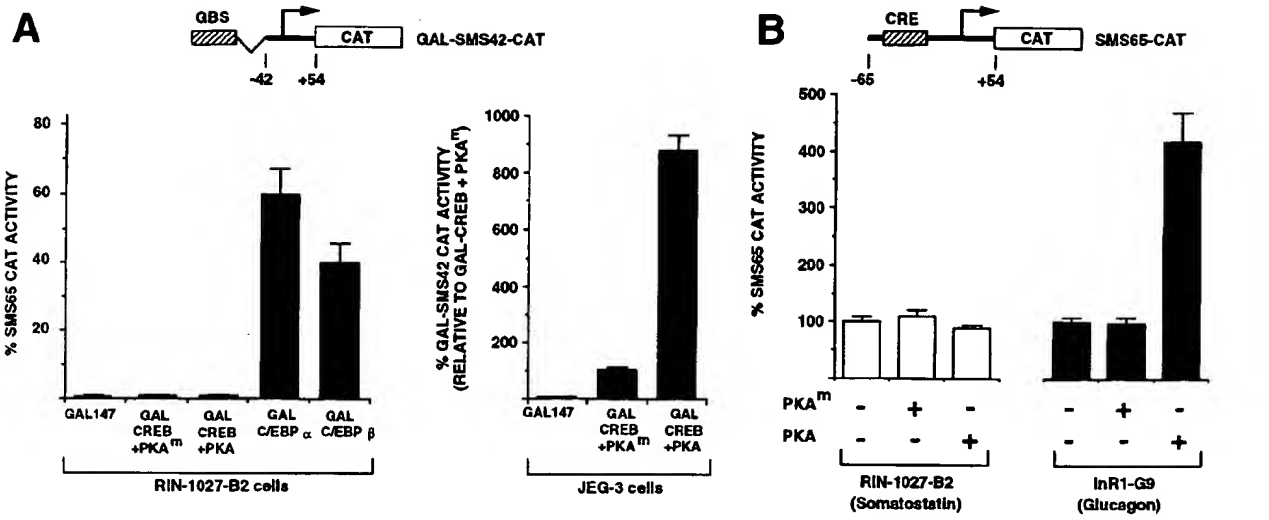
Transfections and CAT assays. Islet cells were transfected by a modified DEAE-dextran procedure (54). JEG-3 cells were transfected by the calcium phosphate precipitation method (14). Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (45) 48 h after transfection. All values are expressed as the means ± standard errors of the means of at least three independent experiments carried out in duplicate.

GAL-CREB consists of rat CREB amino acids 1 to 261 fused to the carboxy terminus of GAL4 (amino acids 1 to 147). For construction of the plasmid encoding GAL-CREB, a PCR fragment with *Bam*HI and *Sac*I linkers was generated with specific primers that anneal to the corresponding sequences of a CREB cDNA template and ligated into the expression plasmid pSG424 (42) in frame with the sequence encoding GAL4 (amino acids 1 to 147), which corresponds to the DNA-binding domain of GAL4. The plasmid encoding GAL-C/EBPβ was constructed in a similar way and contains the entire coding region of a C/EBPβ cDNA. All other plasmids used in transfection studies have been described elsewhere.

DNA-protein binding assays. Electrophoretic mobility shift assays (EMSA) were carried out with nuclear extracts (44) in the presence of the protease inhibitors pepstatin A (1 mg/ml), leupeptin (10 mg/ml), aprotinin (10 mg/ml), and *p*-aminobenzamidine (0.1 mM). Protein concentrations were determined by the Bio-Rad protein assay with bovine serum albumin as a standard. Synthetic complementary oligonucleotides with 5′ GATC overhangs were annealed and labeled by a fill-in reaction, using [α-^{32}P]dATP and Klenow enzyme. Binding reactions were carried out in the presence of 2 μg of poly(dI · dC) and specific competitors, as indicated in the figures, using nuclear extracts (10 μg of protein) incubated with 20,000 cpm of radiolabeled probe (approximately 6 to 10 fmol) in a total volume of 20 μl containing 20 mM potassium phosphate (pH 7.9), 70 mM KCl, 1 mM dithiothreitol, 0.3 mM EDTA, and 10% glycerol. The sequences of the oligonucleotides used are as follows (coding strand): CRE31, 5′-GATCCGG CGCCTCCTTGGCTGACGTCAGAGAGAGAGA-3′; CRE18, 5′-GATCCTT GGCTGACGTCAGAGAGA-3′; APRE-M6, 5′-GATCCACAGTTGTGATT TCACAACCTGACCAGA-3′; and NSC, 5′-GATCCCGGAGGACTGTCCTC CGCGGAGGACTGTCCTCCGA-3′.

Bacterial expression of proteins. Synthesis of recombinant proteins was induced in *Escherichia coli* BL21(DE3)pLysS. CREB was produced from a plasmid consisting of the CREB cDNA placed under the control of the T7 polymerase promoter in the pET-3b prokaryotic expression vector (48, 55). C/EBPβ was produced by the plasmid C/EBPβBD-RSET (gift from D. Ron, New York University) that contains a fragment of the C/EBPβ cDNA spanning the carboxy-terminal domain that includes the bZip region, cloned into the prokaryotic expression vector pRSET-A (Invitrogen) that generates polyhistidine fusion proteins. Recombinant C/EBPβ was purified with a nickel-chelate affinity resin.

Assay of cAMP-dependent protein kinase activity. Nuclei were prepared from B2, InR1-G9, JEG-3, or NIH 3T3 cell monolayers. Cells were scraped in phosphate-buffered saline and collected by centrifugation. The pellet was resuspended in 400 μl of buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9), 10 mM KCl, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors and incubated in ice for 15 min. Nonidet P-40 was added to a final concentration of 0.06%, and then the cells were vortexed vigorously for 10 s. After centrifugation, the supernatant was used for determination of cytoplasmic PKA$_c$ activity. Pelleted nuclei were washed in the same buffer without Nonidet P-40. After the pellet was washed, it was sonicated in 100 μl of the same buffer and centrifuged. PKA activity (41) in the supernatant was determined by incubating 50 μg of protein (determined by the Bio-Rad protein assay) in a volume of 50 μl containing 50 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0), 10 mM $MgCl_2$, 5 mM NaF, 0.25 mg of bovine serum albumin per ml, 0.02 mM [γ-^{32}P]ATP, and 0.1 mg of Kemptide (Sigma) per ml, a PKA$_c$-specific substrate. Reaction mixtures were incubated for



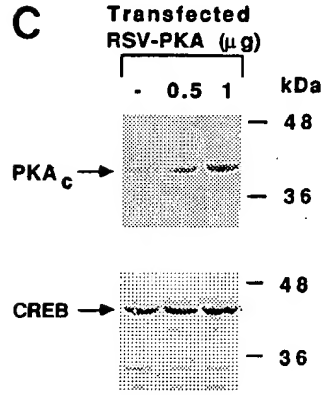


FIG. 3. (A) Relative CAT activities obtained following transient transfections of plasmid GAL-SMS42-CAT into B2 or JEG-3 cells. GAL-SMS42-CAT was cotransfected with an expression plasmid encoding the DNA-binding domain of the yeast activator protein GAL4 (GAL147), a GAL-CREB fusion protein, a GAL-C/EBPα fusion protein, or a GAL-C/EBPβ fusion protein. GAL-CREB was cotransfected with an expression vector encoding either the wild type (PKA) or a mutated version (PKA^m) of the catalytic subunit of cAMP-dependent PKA. Values obtained in experiments involving B2 cells are expressed as percentages of the CAT activities elicited by SMS65-CAT transfected in the same experiments. Because SMS65-CAT is not active in JEG-3 cells, the values are expressed as percentages of the GAL-SMS42-CAT activities elicited by GAL-CREB in the presence of the mutated PKA (PKA^m). A schematic representation of the GAL-SMS42-CAT reporter plasmid is depicted at the top of the panel. (B) Relative CAT activities obtained following transient transfections of somatostatin gene 5′ deletion plasmid SMS65-CAT into islet cell-derived somatostatin-producing B2 or glucagon-producing InR1-G9 cells. SMS65-CAT was transfected alone (−) or cotransfected (+) with a plasmid encoding either a mutated form (PKA^m) or the wild-type form (PKA) of cAMP-dependent PKA. Values are expressed as percentages of the CAT activities elicited by SMS65-CAT transfected alone. A schematic representation of the SMS65-CAT reporter plasmid is depicted at the top of the panel. (C) (Top) Western immunoblot showing the presence of PKA_c in B2 cell extracts before and after transfection of 0.5 or 1 μg of a plasmid encoding PKA_c (RSV-PKA). −, no RSV-PKA. A short exposure (1 min) was used so that the observed increase in immunoreactivity stays within the linear range of detection on the film. Longer exposures (several minutes) revealed readily detectable levels of immunoreactive PKA_c in nontransfected B2 cells (not shown). (Bottom) Western immunoblot of the same extracts with R1090 CREB antiserum, showing the presence of immunoreactive CREB.

10 min at 37°C and spotted onto phosphocellulose filters (Whatman P-81). The filters were washed three times in 75 mM phosphoric acid, and radioactivity was determined by scintillation spectrometry. Values represent means ± standard errors of the means of at least three independent experiments carried out in duplicate.

RESULTS

Transcription of the somatostatin gene in B2 cells is unresponsive to cAMP. Transient transfection studies in B2 cells using SMS65-CAT, a somatostatin-CAT reporter plasmid that contains the CRE as the only active *cis*-acting element (36, 54), showed no observable increase in the levels of CAT activity elicited by SMS65-CAT in response to 8-Br-cAMP (Fig. 1), a finding consistent with those observed earlier (32, 36). As a positive control, CAT activity elicited by SMS65-CAT transfected into another insulinoma cell line (InR1-G9) that produces glucagon is stimulated threefold by 8-Br-cAMP (Fig. 1). We sought to determine the basis for the defective cAMP response in B2 cells.

We considered the possibility that inhibition of the expression of CREB might explain the absence of a transcriptional response of the somatostatin gene to 8-Br-cAMP. We carried out gel EMSA with a synthetic oligonucleotide (CRE31) containing the somatostatin CRE (55) and found that several distinct protein-DNA complexes are present in B2 cells (Fig. 2). The formation of all the complexes detected by EMSA was specifically inhibited by competition with unlabeled CRE oligonucleotides (Fig. 2). Preincubation of the binding reaction mixture with a CREB antiserum (R1090) (58) retarded the migration of protein-DNA complex 2, indicating that CREB is one of the proteins that bind to the somatostatin gene CRE in B2 cells (Fig. 2).

The possibility that the lack of cAMP-dependent transcriptional activity in B2 cells is due to inhibition of the binding of CREB to the CRE by other competing CRE-binding nuclear proteins was investigated by using GAL-CREB, an expression plasmid encoding a fusion protein in which the CRE-binding domain of CREB is replaced by the DNA-binding domain of the *Saccharomyces cerevisiae* transcriptional activator GAL4, that does not bind to any known mammalian gene promoter element. GAL-SMS42-CAT, a CAT reporter plasmid bearing a GAL4-binding site in place of the somatostatin gene CRE, was cotransfected into B2 cells together with expression plasmids encoding either the wild type or an enzymatically inactive

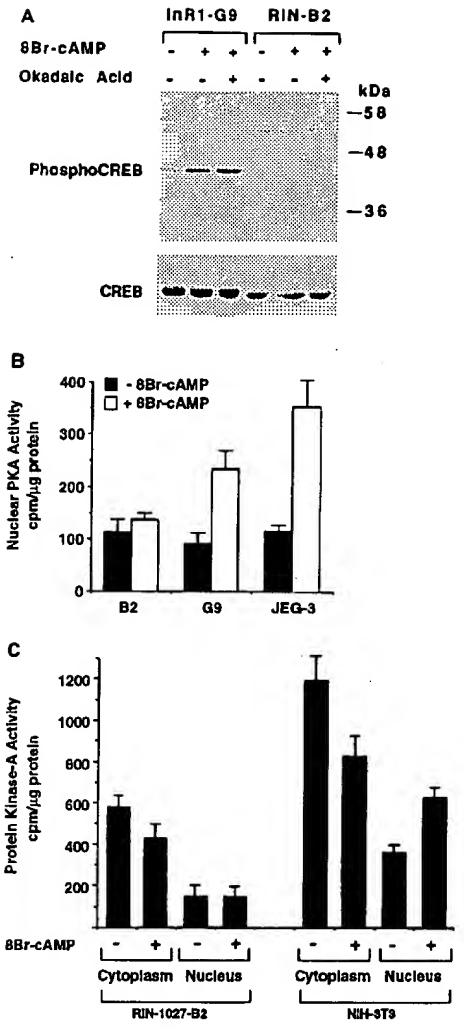


FIG. 4. (A) Western immunoblot carried out with extracts of islet glucagon-producing InR1-G9 or somatostatin-producing B2 cells, prepared before (−) or after (+) treatment of the cells for 30 min with 1 mM 8-Br-cAMP in the presence (+) or absence (−) of 100 nM okadaic acid. The results obtained with an antibody that recognizes specifically phospho-CREB (top) and the results obtained after processing the same extracts with the R1090 antiserum which does not discriminate between CREB and phospho-CREB, (bottom) are shown. (B) Relative PKA enzymatic activities determined for the nuclei of B2, InR1-G9 (G9), or JEG-3 cells prepared before or after treatment of the cells with 1 mM 8-Br-cAMP for 30 min. (C) Relative PKA enzymatic activities determined in the cytoplasm and nuclei of B2 or NIH 3T3 cells prepared before (−) or after (+) treatment of cells with 1 mM 8-Br-cAMP for 30 min. All reactions were carried out in the presence of 10 μM cAMP to achieve maximum PKA activity.

mutant of the β isoform of the PKA_c (plasmids RSV-PKA and RSV-PKA^m, respectively) (25). No enhancement of CAT activity was observed when GAL-CREB was cotransfected with either mutated or wild-type PKA_c, indicating that the transactivational functions of CREB are impaired in B2 cells (Fig. 3A). As a positive control, the transactivation of GAL-SMS42-CAT is stimulated eightfold by GAL-CREB and PKA_c in JEG-3 choriocarcinoma cells (Fig. 3A). To determine whether B2 cells may provide a permissive environment for the expression of GAL-SMS42-CAT, GAL-SMS42-CAT was cotransfected with expression plasmids encoding fusion proteins consisting of the GAL4-binding domain and the transactivation domains of transcription factor C/EBPα (33) or C/EBPβ. GAL-SMS42-CAT is transactivated by both GAL-C/EBPα and GAL-C/EBPβ in B2 cells (Fig. 3A). Thus, the failure of CREB to activate somatostatin gene transcription in B2 cells is not due to competitive interference with other CRE-binding proteins. Rather, the transactivation domain of CREB appears not to function, raising the possibility of a defect in the cAMP signaling pathway and consequent lack of phosphorylation of CREB by PKA.

The existence, however, of a defect within proximal steps in the cAMP-dependent signal transduction pathway in B2 cells appears unlikely, because hormonal stimulation of these cells rapidly increases levels of cAMP (11). Therefore, we examined the possibility that more-distal steps in the cAMP-dependent signaling pathway involving PKA itself may be defective. Transient transactivation studies using the somatostatin-CAT reporter plasmid SMS65-CAT and expression plasmids RSV-PKA^m and RSV-PKA were done in B2 cells compared with control InR1-G9 cells. In B2 cells, the levels of CAT activity were detectable, but cotransfection with the PKA_c expression plasmid (Fig. 3B) or with an expression plasmid encoding a mutated catalytic subunit of PKA that does not interact with the regulatory subunits (29) (data not shown) failed to increase activity further. In contrast, in InR1-G9 cells cotransfection of SMS65-CAT with RSV-PKA resulted in a fourfold increase in CAT activity (Fig. 3B). The activities of SMS65-CAT in B2 and InR1-G9 cells are similar (24% ± 1.3% and 22% ± 0.9%, respectively, relative to the activity of the control plasmid RSV-CAT transfected in each cell type). PKA_c is expressed by the plasmid RSV-PKA in transfected B2 cells as monitored by Western immunoblotting (Fig. 3C). CREB is readily detected by Western immunoblots of the extracts of B2 (Fig. 3C) and InR1-G9 (not shown) cells, using the CREB antiserum R1090, indicating that CREB is expressed equivalently in the two islet cell lines.

CREB is not phosphorylated by PKA in B2 cells. Because the transcriptional activity of CREB is critically dependent upon phosphorylation by cAMP-activated PKA (13), we investigated whether the failure of CREB to transactivate the somatostatin gene promoter may be due to defective phosphorylation by PKA. To determine whether CREB is phosphorylated by PKA in B2 cells, we treated cells with 8-Br-cAMP in the absence or presence of okadaic acid, a phosphatase inhibitor that inhibits dephosphorylation of CREB (16), and assayed for CREB phosphorylation by Western immunoblotting with an antibody specifically directed against phospho-CREB (12). Incubation of InR1-G9 cells with 8-Br-cAMP for 30 min results in the phosphorylation of CREB, further enhanced by okadaic acid (Fig. 4A). In contrast, no phospho-CREB is detected in extracts of similarly treated B2 cells, indicating a failure of cAMP stimulation to phosphorylate CREB (Fig. 4A). The amounts of CREB (phospho- and dephospho-) in B2 and InR1-G9 cells are comparable, as determined by immunoblotting using an antiserum (R1090) that detects both phospho- and dephospho-CREB equivalently (Fig. 4A).

Since Western immunoblot analyses showed that the amounts of the catalytic subunit of PKA in extracts of B2 and InR1-G9 cells are comparable (data not shown), the failure to detect phospho-CREB in B2 cells suggested that the catalytic activity of PKA may be defective. Because the activation of CREB by PKA phosphorylation occurs in the nucleus after cAMP-mediated translocation of PKA_c from the cytoplasm to the nucleus, we assayed directly for PKA_c catalytic activities in nuclear extracts prepared from B2 cells and compared them with the activities obtained in InR1-G9 and JEG-3 cells. Treat-

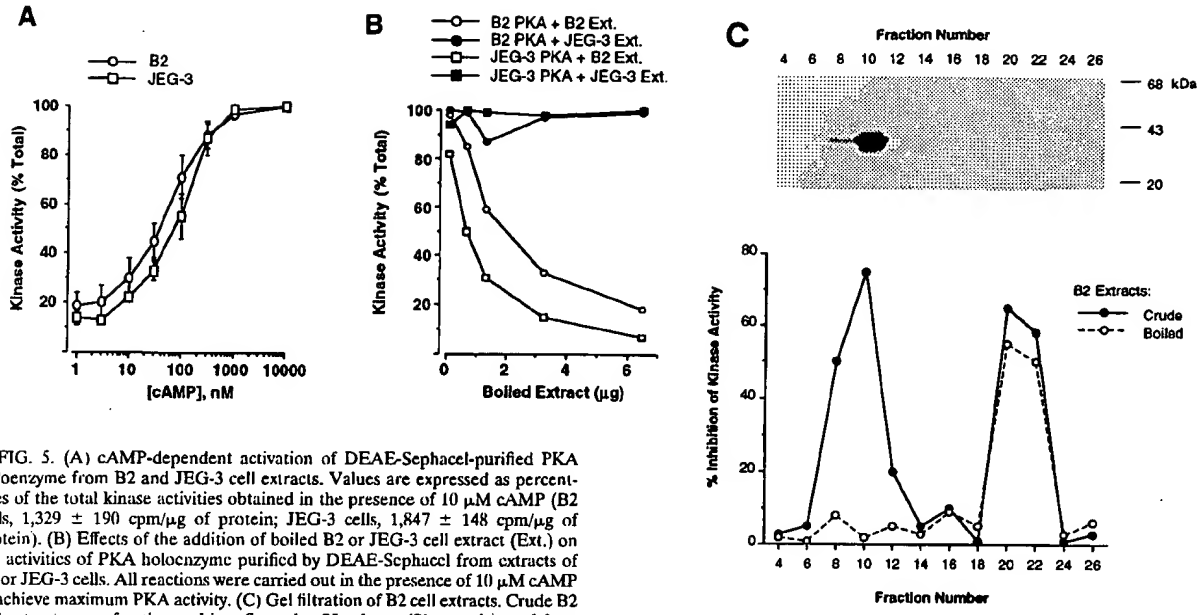


FIG. 5. (A) cAMP-dependent activation of DEAE-Sephacel-purified PKA holoenzyme from B2 and JEG-3 cell extracts. Values are expressed as percentages of the total kinase activities obtained in the presence of 10 µM cAMP (B2 cells, 1,329 ± 190 cpm/µg of protein; JEG-3 cells, 1,847 ± 148 cpm/µg of protein). (B) Effects of the addition of boiled B2 or JEG-3 cell extract (Ext.) on the activities of PKA holoenzyme purified by DEAE-Sephacel from extracts of B2 or JEG-3 cells. All reactions were carried out in the presence of 10 µM cAMP to achieve maximum PKA activity. (C) Gel filtration of B2 cell extracts. Crude B2 cell extracts were fractionated in a Superdex-75 column (Pharmacia), and fractions were collected. (Top) One aliquot of each fraction was labeled with ^{32}P-8-N_3-cAMP under UV radiation, resolved by SDS-PAGE, and exposed for autoradiography. This procedure allowed the identification of the photoaffinity-labeled regulatory subunit of PKA in fractions 8 and 10. (Bottom) Fractions from crude or boiled B2 cell extracts were assayed for PKA inhibitory activity with purified catalytic subunit (Promega). Experiments were run in triplicate and were repeated three times with similar results.

ment of control InR1-G9 and JEG-3 cells with 1 mM 8-Br-cAMP for 30 min resulted in 2.5- and 3.5-fold increases in the catalytic activity of PKA_c, respectively, whereas in B2 cells PKA_c catalytic activity did not increase in response to treatment with 8-Br-cAMP (Fig. 4B). Thus, a deficiency in nuclear PKA activity in B2 cells appears to be the cause of the lack of phosphorylation of CREB in response to 8-Br-cAMP.

We next compared the relative PKA_c activities in the cytoplasm and nuclei of B2 and NIH 3T3 cells. We chose NIH 3T3 as the control cells because phosphorylation of CREB after translocation of PKA_c from the cytoplasm to the nucleus occurs in these cells in response to cAMP stimulation (17). We determined PKA_c activity in the presence of 10 µM cAMP to ensure that the regulatory and catalytic subunits of PKA are dissociated, so that the maximum activities in cytoplasm or nucleus are measured. As shown in Fig. 4C, treatment of NIH 3T3 cells with 1 mM 8-Br-cAMP for 30 min resulted in a decrease in the total catalytic activity of PKA_c in the cytoplasm and a concomitant increase in nuclear activity, reflecting the translocation of PKA_c into the nuclear compartment. The catalytic activity of PKA_c in the cytoplasm of untreated B2 cells was significantly lower than in untreated NIH 3T3 cells (Fig. 4C). Treatment of B2 cells with 8-Br-cAMP resulted in a decrease in the catalytic activity of PKA_c in the cytoplasm. However, no concomitant increase in PKA_c activity was observed in nuclear extracts of treated B2 cells (Fig. 4C). If B2 cells had a defect in translocation of PKA_c, a decrease in cytoplasmic catalytic activity should not have occurred. Therefore, the results of these experiments suggest the presence, predominantly in the nuclear compartment of B2 cells, of an inhibitor of PKA_c.

To explore this possibility in further detail, we subjected B2 cell extracts to ion-exchange chromatography on a DEAE-Sephacel column (15) in an attempt to separate the PKA holoenzyme from the putative inhibitor. Prior to chromatography, PKA_c was readily detectable by Western immunoblotting of crude extracts of B2 cells, but its activity was not detectable even in the presence of 10 µM cAMP (not shown). However, after ion-exchange chromatography, the addition of increasing amounts of cAMP to the fractions containing the purified PKA holoenzyme resulted in increased PKA_c activities in a concentration-dependent manner that was indistinguishable from that observed with similarly purified PKA holoenzyme from JEG-3 cells (Fig. 5A). Confirmation of direct binding of cAMP to the regulatory subunits of PKA was obtained by incubation of B2 cell extracts with ^{32}P-8-N_3-cAMP, followed by UV radiation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (47), which showed the specific radiolabeling of the regulatory subunits (data not shown). These experiments indicate that B2 cells have normal PKA holoenzymes that can be purified by ion-exchange chromatography and activated by cAMP through binding to the regulatory subunits. Next, we added increasing amounts of boiled B2 cell extracts to the purified PKA holoenzyme in the presence of 10 µM cAMP, which resulted in inhibition of cAMP-induced PKA_c activities (Fig. 5B). This effect was not observed when boiled extracts of control JEG-3 cells were added (Fig. 5B).

To further characterize this inhibitory activity biochemically, we fractionated B2 cell extracts by gel filtration on a Superdex-75 column (Pharmacia) and assayed each fraction for inhibition of the catalytic activity of PKA_c (Promega) in vitro. When crude B2 cell extracts were used, two peaks of PKA_c inhibitory activity were observed (Fig. 5C). These activities were observed to coelute with molecular mass standards corresponding to 40 and 10 kDa, respectively. The first peak of inhibitory activity (fractions 8 to 10) was undetectable when boiled B2 cell extracts were used, but the activity of the second peak (fractions 20 to 22) was found to be heat stable (Fig. 5C). The heat instability and the approximate molecular mass of the activity present in the first peak suggested that the regulatory subunit of PKA may be responsible for this activity. To test this notion, aliquots of all column fractions were incubated with

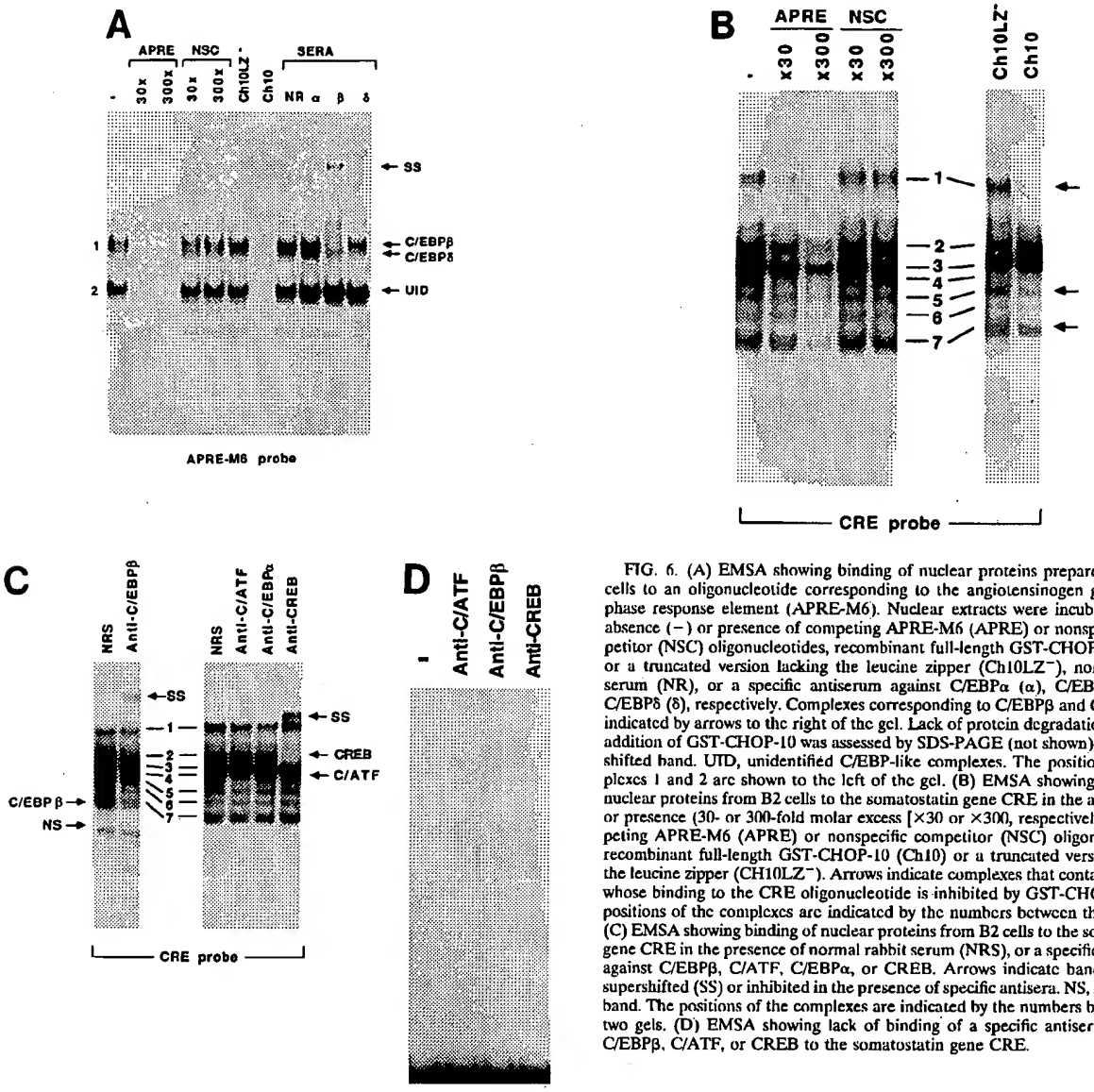


FIG. 6. (A) EMSA showing binding of nuclear proteins prepared from B2 cells to an oligonucleotide corresponding to the angiotensinogen gene acute-phase response element (APRE-M6). Nuclear extracts were incubated in the absence (−) or presence of competing APRE-M6 (APRE) or nonspecific competitor (NSC) oligonucleotides, recombinant full-length GST-CHOP-10 (Ch10) or a truncated version lacking the leucine zipper (Ch10LZ⁻), normal rabbit serum (NR), or a specific antiserum against C/EBPα (α), C/EBPβ (β), or C/EBPδ (δ), respectively. Complexes corresponding to C/EBPβ and C/EBPδ are indicated by arrows to the right of the gel. Lack of protein degradation after the addition of GST-CHOP-10 was assessed by SDS-PAGE (not shown). SS, supershifted band. UID, unidentified C/EBP-like complexes. The positions of complexes 1 and 2 are shown to the left of the gel. (B) EMSA showing binding of nuclear proteins from B2 cells to the somatostatin gene CRE in the absence (−) or presence (30- or 300-fold molar excess [×30 or ×300, respectively]) of competing APRE-M6 (APRE) or nonspecific competitor (NSC) oligonucleotides, recombinant full-length GST-CHOP-10 (Ch10) or a truncated version lacking the leucine zipper (CH10LZ⁻). Arrows indicate complexes that contain proteins whose binding to the CRE oligonucleotide is inhibited by GST-CHOP-10. The positions of the complexes are indicated by the numbers between the two gels. (C) EMSA showing binding of nuclear proteins from B2 cells to the somatostatin gene CRE in the presence of normal rabbit serum (NRS), or a specific antiserum against C/EBPβ, C/ATF, C/EBPα, or CREB. Arrows indicate bands that are supershifted (SS) or inhibited in the presence of specific antisera. NS, nonspecific band. The positions of the complexes are indicated by the numbers between the two gels. (D) EMSA showing lack of binding of a specific antiserum against C/EBPβ, C/ATF, or CREB to the somatostatin gene CRE.

^{32}P-8-N_3-cAMP, radiated with UV light, and resolved by SDS-PAGE (47). Photoaffinity labeling with ^{32}P-8-N_3-cAMP revealed the presence of regulatory subunits of PKA only in fractions 8 to 10 of crude B2 cell extracts (Fig. 5C). No incorporation of ^{32}P-8-N_3-cAMP was observed when photoaffinity labeling was carried out in the presence of 40 μM cAMP (not shown), indicating specificity of binding of ^{32}P-8-N_3-cAMP to the regulatory subunits of PKA. Thus, these experiments indicate that in addition to regulatory subunits of PKA that normally inhibit the activity of PKA in the absence of cAMP stimulation, B2 cells contain a lower-molecular-weight, heat-stable inhibitor that inhibits PKA even in the presence of cAMP.

C/EBPs in B2 cells bind to the somatostatin gene CRE. The experiments described above suggested that proteins other than CREB bind the CRE and activate constitutive levels of transcription observed after transfection. Most CRE-binding proteins belong to the CREB/ATF family of transcription factors characterized by conserved bZip domains that mediate DNA binding and protein dimerization (for a recent review, see reference 27). Proteins of the related but different C/EBP family of bZip transcription factors also bind to CRE sites (3, 30, 31, 56), but the functional significance of these bindings is unknown. Therefore, we examined the possibility that some of the somatostatin CRE-binding proteins detected by EMSA are related to C/EBP.

To determine whether C/EBP-like proteins are expressed in somatostatin-producing pancreatic islet cells, we tested whether an oligonucleotide probe corresponding to the angiotensinogen gene acute-phase response element (APRE-M6), a well-characterized C/EBP-binding site (5), binds to proteins present in the nuclear extracts of B2 cells. Two major sequence-specific DNA-protein complexes were detected with the APRE-M6 probe by EMSA (Fig. 6A). The addition of recombinant protein CHOP-10, an inhibitor of the binding of C/EBP to DNA (40), to the binding reaction mixture resulted in the inhibition of binding of protein complexes to the APRE-M6 probe, indicating that these complexes are com-

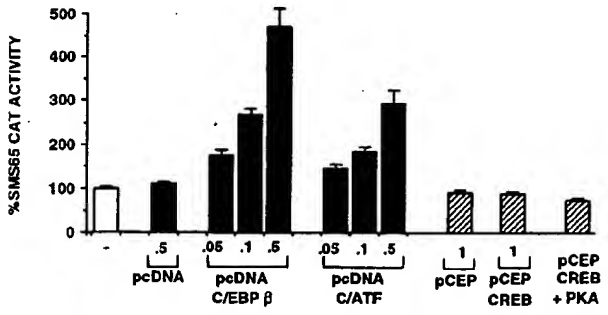


FIG. 7. Relative CAT activities obtained following transient transfections of somatostatin gene 5′ deletion plasmid SMS65-CAT into islet cell-derived somatostatin-producing B2 cells. SMS65-CAT was transfected alone (−) or cotransfected with increasing amounts (in micrograms) of an expression plasmid (pcDNA or pCEP) encoding either C/CBPβ, C/ATF, or CREB. Values are expressed as percentages of the CAT activities elicited by SMS65-CAT transfected alone.

posed of C/EBP-related proteins (Fig. 6A). Attenuation of the labeled complexes was observed after the addition of an amount of competitor oligonucleotide as low as the 10-fold molar ratio, indicating a relatively high binding affinity as well as specificity. Two members of the C/EBP family, C/EBPβ and C/EBPδ, were identified as components of complex 1 by preincubation of the binding reaction mixtures with specific antisera to these transcription factors. The presence of these antisera resulted in the appearance of a supershifted complex (C/EBPβ) and inhibition of binding of another protein complex (C/EBPδ), respectively (Fig. 6A). No C/EBPα was detected in these complexes with a specific C/EBPα antiserum (Fig. 6A). In addition, C/EBPα was undetectable in B2 cells by Western immunoblot and immunoprecipitation studies, but it was readily detectable after transfections of B2 cells with an expression vector encoding C/EBPα (data not shown).

C/EBP proteins in B2 cells are part of the protein complexes recognized by the somatostatin CRE, as shown by EMSA (Fig. 6B and C). The C/EBP-binding APRE-M6 oligonucleotide competed with CRE-bound complexes 1, 4, 6, and 7, whereas the addition of CHOP-10 to the binding reaction mixture eliminated complex 1 and attenuated the relative intensities of complexes 5 and 7 (Fig. 6B). C/EBPβ antiserum supershifted complex 7 (Fig. 6C). No supershift was observed after the addition of C/EBPα (Fig. 6C) or C/EBPδ (data not shown) antiserum. Therefore, C/EBPβ is present in B2 cells and binds to the somatostatin gene CRE.

We identified previously a new member of the CREB/ATF family of transcription factors, C/ATF, that dimerizes with C/EBP proteins and directs their binding to CRE sites (56). C/ATF antiserum attenuated EMSA complex 4, indicating that this complex contains C/ATF bound to the CRE (Fig. 6C). Because the C/ATF and C/EBPβ antisera interfered with complexes with different electrophoretic mobilities, the complexes probably do not correspond to C/ATF-C/EBPβ heterodimers (56). None of the antisera used in these experiments were found to bind to the CRE oligonucleotide probe (Fig. 6D). C/ATF in B2 cells was also detected by Western immunoblot analysis (data not shown).

C/EBPβ and C/ATF activate and CREB represses transcription from the somatostatin CRE. Having established that C/EBPβ and C/ATF in B2 cells bind the somatostatin gene CRE, we sought to determine whether these transcription factors transactivate the somatostatin gene promoter via the CRE site. Transient transactivation assays were done in B2 cells with SMS65-CAT as a *cis*-reporter plasmid and different amounts of *trans*-expression plasmids encoding C/EBPβ or C/ATF, resulting in dose-dependent increases of CAT activity (Fig. 7). Transactivation of SMS65-CAT by C/EBPβ was more efficient than transactivation by C/ATF (Fig. 7) and was not modified by treatment of B2 cells with 8-Br-cAMP or by cotransfection with RSV-PKA (data not shown). When both C/ATF and C/EBPβ expression vectors were cotransfected together with SMS65-CAT, additive effects were observed (data not shown). Cotransfection of SMS65-CAT with an expression plasmid encoding CREB did not increase CAT activity even after cotransfection with the RSV-PKA expression plasmid (Fig. 7).

Both C/EBPβ and CREB bind to the CRE of the somatostatin gene, suggesting that they may compete for binding to the CRE. Thus, we examined by EMSA the binding of a truncated bacterially expressed C/EBPβ (containing only the C-terminal 145 amino acids corresponding to the bZip domain) and a bacterially expressed full-length CREB to labeled somatostatin CRE oligonucleotide. Binding of both proteins to the CRE was abolished after the addition of similar amounts of excess CRE oligonucleotide (Fig. 8A), indicating that competition of C/EBPβ and CREB for binding to the CRE can occur. We tested this notion directly by adding increasing amounts of CREB to a constant amount of C/EBPβ bound to the CRE. Doing this resulted in a loss of the gel-shifting activity contributed by C/EBPβ and an increase in the CREB gel-shifting activity (Fig. 8B). Experiments were also carried out by using the APRE-M6 oligonucleotide as the probe. CREB did not bind to the APRE-M6 probe, and no diminution of C/EBPβ binding to the APRE-M6 was observed in the presence of CREB (Fig. 8B), demonstrating that the displacement of the binding of C/EBPβ to the CRE by CREB is specific for the CRE sequence.

We next sought to determine whether displacement of C/EBPβ from the CRE by an excess of dephospho-CREB would down-regulate somatostatin gene transcription. B2 cells were cotransfected with SMS65-CAT and a C/EBPβ expression plasmid in the absence or presence of increasing amounts of an expression plasmid encoding CREB. C/EBPβ-induced SMS65-CAT transcription decreased with increasing amounts of CREB expression plasmid (Fig. 8C). As a control, B2 cells were cotransfected with the reporter plasmid GAL-SMS42-CAT, an expression plasmid encoding a GAL-C/EBPβ fusion protein, and increasing amounts of CREB expression plasmid. No significant decrease in GAL-SMS42-CAT activity was observed (Fig. 8D), indicating that the effect of CREB on C/EBPβ-induced SMS65-CAT transcription is due to competition of C/EBPβ by CREB bound to the CRE and not to sequestration of coactivating factors (squelching) or to interfering interactions between C/EBPβ and CREB transactivation domains. These experiments indicate that in B2 cells CREB represses somatostatin gene expression by inhibiting by competition other transactivating transcription factors bound to the CRE and suggest that basal transcriptional levels of SMS65-CAT result from an equilibrium between C/EBP transactivators and dephospho-CREB repressor.

DISCUSSION

These studies demonstrate that transcription mediated by the somatostatin gene CRE in somatostatin-producing B2 cells is repressed by CREB because of a defect in the cAMP-dependent signaling pathway that inhibits phosphorylation of CREB by PKA. Somatostatin gene expression is responsive to cAMP stimulation in rat pancreatic islets (32). The islet B2 clonal cell line, however, used in this study was derived from a

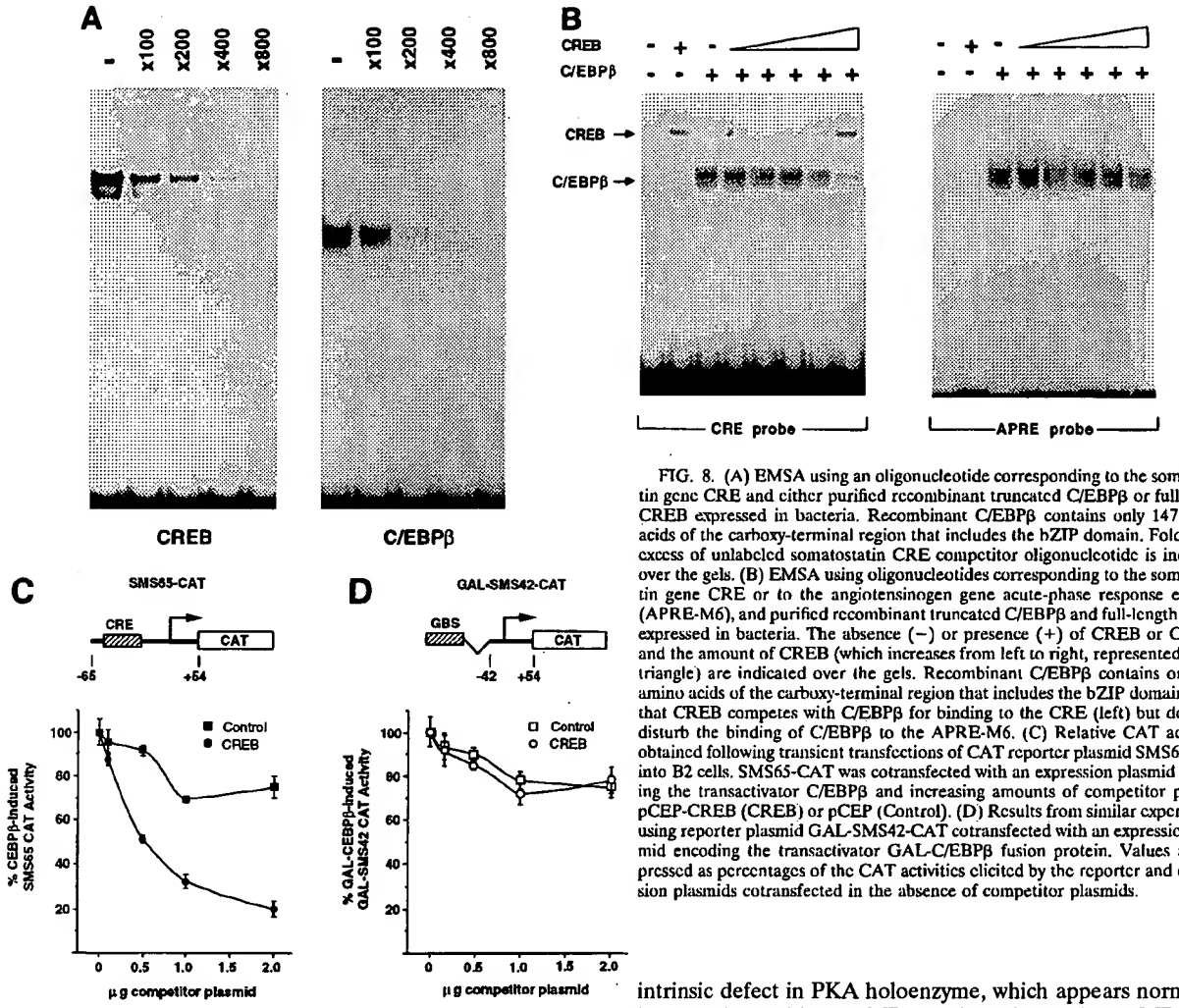


FIG. 8. (A) EMSA using an oligonucleotide corresponding to the somatostatin gene CRE and either purified recombinant truncated C/EBPβ or full-length CREB expressed in bacteria. Recombinant C/EBPβ contains only 147 amino acids of the carboxy-terminal region that includes the bZIP domain. Fold molar excess of unlabeled somatostatin CRE competitor oligonucleotide is indicated over the gels. (B) EMSA using oligonucleotides corresponding to the somatostatin gene CRE or to the angiotensinogen gene acute-phase response element (APRE-M6), and purified recombinant truncated C/EBPβ and full-length CREB expressed in bacteria. The absence (−) or presence (+) of CREB or C/EBPβ and the amount of CREB (which increases from left to right, represented by the triangle) are indicated over the gels. Recombinant C/EBPβ contains only 147 amino acids of the carboxy-terminal region that includes the bZIP domain. Note that CREB competes with C/EBPβ for binding to the CRE (left) but does not disturb the binding of C/EBPβ to the APRE-M6. (C) Relative CAT activities obtained following transient transfections of CAT reporter plasmid SMS65-CAT into B2 cells. SMS65-CAT was cotransfected with an expression plasmid encoding the transactivator C/EBPβ and increasing amounts of competitor plasmid pCEP-CREB (CREB) or pCEP (Control). (D) Results from similar experiments using reporter plasmid GAL-SMS42-CAT cotransfected with an expression plasmid encoding the transactivator GAL-C/EBPβ fusion protein. Values are expressed as percentages of the CAT activities elicited by the reporter and expression plasmids cotransfected in the absence of competitor plasmids.

radiation-induced insulinoma (34). The process of radiation-induced cellular transformation may have deregulated the expression of the somatostatin gene and perhaps other genes otherwise under the control of the cAMP signaling pathway. The state of relative dedifferentiation of the B2 cells may correspond to a stage of constitutive somatostatin gene expression that occurs during fetal islet development (1). Such a phenotypical switch from a cAMP-regulated pathway to a constitutive pathway of gene expression may be important for the maintenance of cell proliferation at particular times during development. Consistent with this view are the observations that somatostatin gene expression is not dependent on cAMP stimulation in at least two other pancreatic cell lines, RIN-1056-A (53a) and Tu6 cells, in which lack of cAMP-induced phosphorylation of CREB has been observed (22). In addition, in transfected F9 cells, cAMP inducibility of the somatostatin gene promoter appears to be dependent on the state of differentiation of the cells (24).

We observe that the absence of CREB phosphorylation is due to lack of cAMP-induced PKA catalytic activity in the nuclei of B2 cells. This lack of activation is not due to an intrinsic defect in PKA holoenzyme, which appears normal in its capacity to bind cAMP and is activated by cAMP after purification from B2 cell extracts. In addition, it is unlikely that lack of activation of PKA results from sequestration of the catalytic subunit in a cytoplasmic compartment due to unusually high levels of regulatory subunit of PKA, because cotransfection of B2 cells with the SMS65-CAT reporter plasmid and an expression plasmid encoding a mutated catalytic subunit of PKA that does not interact with the regulatory subunits (29) does not result in increased levels of CAT activity. Rather, lack of activation of PKA appears to be due to the presence of a heat-stable inhibitor of PKA (PKI).

Our studies indicate that PKA inhibitors in B2 cells have important functions in transcriptional regulation of cAMP-dependent gene expression. Recently, molecular characterizations of cDNAs encoding at least three different PKI isoforms (PKIα, PKIβ1, and PKIβ2) have been reported (43, 57). Both PKIα and PKIβ are expressed in pancreatic tissue (57), and it is not clear whether the PKI activity identified in B2 cells in this study corresponds to any of the known isoforms of PKI. Earlier biochemical studies revealed the presence of different size forms (between 15 and 4 kDa) of PKI in several tissues (43 and 57), but the exact number of PKI isoforms and their physiological roles remain obscure. We are currently carrying out experiments to determine unequivocally whether PKI from B2

cells corresponds to a new isoform in order to proceed to the molecular cloning of its cDNA.

Earlier studies showing that a mutated form of CREB with a serine-to-alanine substitution at position 133 (that is phosphorylated by PKA_c) is a negative transcriptional regulator of the *c-jun* proto-oncogene promoter (21) suggested that dephospho-CREB acts as a repressor of gene transcription. Our observations confirm this notion and underscore the critical dependency of the transactivation functions of CREB on phosphorylation by PKA (or calcium-dependent kinases [46]). Dephospho-CREB is a potent negative regulator of CRE-mediated transcription in B2 cells, in which the cAMP-PKA pathway is defective, which has implications for CREB-regulated gene transcription in cells in which this pathway is intact. It seems clear that the relative ratios of nuclear dephospho- and phospho-CREB determine the relative transcriptional transactivational potency of CREB. This supposition is consistent with the recently reported findings of Loriaux et al. (23), in which CREB dimers consisting of one phosphorylated monomer and one mutated unphosphorylated monomer, gives 50% of the transactivational activity of the wild-type phosphorylated dimer.

The lack of phosphorylation of CREB, however, does not fully explain the lack of basal activity of CREB, because CREB has transcriptional transactivation domains that act in the absence of phosphorylation by PKA (6, 22, 37). Therefore, an additional defect may be present in B2 cells, perhaps involving adapter proteins associated with the basal transcription machinery. The report of a CREB-binding protein that interacts with the kinase-inducible domain of CREB (8) suggests that interactions between CREB and adapter proteins may be important for the transcriptional transactivation of CREB. In addition, our studies do not strictly rule out the possibility that other factors such a hyperactive phosphatases or excess PKA regulatory subunits (4) may also contribute to lack of cAMP-induced transcriptional activity in B2 cells.

Recombinant C/EBP binds efficiently to CRE sequences in vitro (3, 31, 56). C/EBPβ regulates phosphoenolpyruvate carboxykinase gene transcription by binding to several sites including an asymmetric CRE (30). We extend these observations by showing that at least two C/EBP proteins, C/EBPβ and C/EBPδ, are expressed in somatostatin-producing B2 cells and bind the somatostatin gene CRE and that C/EBPβ transactivates the somatostatin gene promoter. Our findings point to the existence of additional unidentified C/EBP-like proteins in B2 cells, as in other cell types (5, 40), that bind to the somatostatin CRE.

C/EBPα is typically expressed in cells upon reaching terminal differentiation (52). C/EBPβ, however, is expressed in proliferating cells as an effector protein activated by intracellular signaling pathways (50, 59), including the cAMP-dependent pathway in PC12 cells (26). In our studies, however, C/EBPβ activated transcription from the somatostatin gene CRE, but no further enhancement of this effect by 8-Br-cAMP or by cotransfection with RSV-PKA was observed. This lack of response to cAMP may be due to differences between PC12 and B2 cells or may be related to the defective phosphorylation of CREB by PKA.

In earlier studies (56), we described C/ATF, a bZip protein that mediates functional cross talk between transcription factors of both ATF and C/EBP families by forming heterodimers with C/EBPβ. In this study, we find immunoreactive C/ATF in somatostatin-producing B2 cells. C/ATF regulates the expression of the somatostatin gene in B2 cells by binding to the CRE. However, the immunoreactive complex detected with the C/ATF antiserum by EMSA (complex 4) is different from the immunoreactive complex detected with the C/EBPβ antiserum (complex 7). Furthermore, only additive, not synergistic, interactions between C/ATF and C/EBPβ were detected in cotransfection experiments with SMS65-CAT. Therefore, C/ATF and C/EBPβ do not appear to interact in B2 cells, as they do in vitro or in transfected HepG2 cells (56).

In summary, we show that transcription from the somatostatin gene CRE in islet B2 cells is the result of a complex interaction between positive- and negative-acting bZip transcription factors, some of which remain to be identified. Further, these observations emphasize the critical importance of the availability of nuclear PKA_c activity in determining the balance between activation and repression of CRE-mediated transcription by phospho- and dephospho-CREB, respectively. Alterations in the cAMP second messenger signaling pathway and the existence of cross talk between different families of CRE-binding bZip transcription factors may have important functional consequences for developmental gene expression, switching from a regulated to constitutive pattern.

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Thank you

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THE PROTOONCOGENE C-FOS IS INDUCED BY CORTICOTROPIN-RELEASING FACTOR AND STIMULATES PROOPIOMELANOCORTIN GENE TRANSCRIPTION IN PITUITARY CELLS

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The Protooncogene c-*fos* Is Induced by Corticotropin-Releasing Factor and Stimulates Proopiomelanocortin Gene Transcription in Pituitary Cells

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CRF is a potent hypophysiotropic factor which stimulates POMC-producing cells in both the intermediate and anterior pituitary. Although its secretagogue effects and its stimulatory action on POMC gene expression are well documented, the mechanisms by which CRF modulates gene regulation are poorly understood. In this study we have investigated the mechanisms by which CRF stimulates the immediate early gene c-*fos*. Studies were performed in the corticotroph-derived AtT20 cell line. We show that CRF induces a transient increase in c-*fos* mRNA levels. This induction is reduced by blockade of calcium entry and by calmodulin inhibitors, suggesting that the CRF-induced c-*fos* increase is mediated in part by the second messenger Ca^{2+} and the Ca^{2+}/calmodulin kinase. When protein kinase-A (PKA) was inhibited by introduction of a mutated regulatory subunit of PKA that lacks cAMP-binding sites, the stimulation of c-*fos* mRNA by CRF was abolished. Taken together, these results suggest that CRF activates the c-*fos* protooncogene via PKA and the Ca^{2+}/calmodulin kinase. These results were confirmed and extended by gene transfer studies using chimera genes containing c-*fos* promoter sequences coupled to the chloramphenicol acetyl transferase reporter gene. This series of experiments shows that CRF stimulates c-*fos* transcription by mechanisms requiring PKA activation.

Furthermore, cotransfection experiments with the POMC promoter linked to the chloramphenicol acetyl transferase reporter gene along with an expression vector coding for *cFOS* showed efficient stimulation of POMC gene transcription by *cFOS*.

In summary, c-*fos* mRNA accumulation is an early genomic signal in pituitary cells in response to CRF, and *cFOS* may represent a signal controlling POMC gene expression. (Molecular Endocrinology 5: 1301–1310, 1991)

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Molecular Endocrinology

INTRODUCTION

The 41-amino acid peptide CRF has potent stimulatory effects on POMC-producing cells from both the intermediate lobe (IL) and the anterior lobe (AL) of the pituitary (for review, see Ref. 1). The AtT20 pituitary tumor cell line, derived from mouse anterior pituitary tissue, has provided an interesting model system for studying the molecular mechanisms underlying the stimulatory effects of CRF. The latter include increased secretion of POMC-derived peptides and, at the genomic level, the stimulation of POMC gene transcription (2) and POMC mRNA accumulation (3–5).

Biochemical studies have shown that CRF activates adenylate cyclase (probably via the GTP-binding protein Gs), thereby increasing intracellular cAMP levels (6). Thus, cAMP is one possible link or effector in the regulatory cascade via protein kinase-A (PKA) that may affect peptide secretion and POMC gene expression. This was further suggested by experiments in which PKA was blocked by introduction of PKA inhibitors with loaded liposomes (5). This treatment reduced the CRF-stimulated POMC mRNA increase. On the other hand, hormonal secretagogues that stimulate POMC-derived peptide release and cAMP levels also increase (directly or secondary to cAMP increase) cytosolic free calcium levels ($[Ca]_i$) (7). The source of increased calcium appears to be extracellular. Indeed, the rise in $[Ca]_i$ and the increase in secretion (8) and POMC mRNA accumulation (4) after stimulation by CRF are inhibited by pretreatment with inorganic (cadmium, cobalt, *etc.*) and organic (D600 and nifedipine) blockers of voltage-dependent calcium channels.

In contrast, little is known about the effects of CRF on gene regulation. It is well established that this hormone acts on POMC gene expression by increasing the rate of transcription and accumulation of POMC mRNA in melanotrophs and corticotrophs (see Ref. 9 and references therein). At the present time, the molecular mechanisms and the transacting factor(s) that mediate this effect have not been characterized. Stimulation of POMC gene transcription could be mediated by posttranslational modifications (12) (*e.g.* phosphorylation) initiated by changes in second messenger levels (cAMP and Ca^{2+}) generated by CRF. Alternatively, CRF could influence hormonal synthesis by inducing *de novo* synthesis of transacting factors. This possibility would include the involvement of early immediate genes inducible by cAMP and Ca^{2+}. Such induction mechanisms have been particularly well studied in electrically excitable cells for the protooncogene c-*fos*. This gene is rapidly induced by a variety of extracellular signals (growth factors, neurotransmitters, or depolarizing agents) (13–16) and is under the control of the cAMP and Ca^{2+} second messenger pathways (14, 17–19). Importantly, it is clear that c-*fos* is not only involved in processes controlling cell growth, but is also induced in cells in which the mitotic program has been stopped. Furthermore, c*FOS* has been shown to regulate the expression of a variety of genes, including neuropeptides (20–22), and a similar control could be exerted on the POMC gene in pituitary cells

To test whether c-*fos* is implicated in the regulatory pathways controlled by CRF in pituitary cells, we have studied its induction in a corticotroph-derived cell line. In this report we show 1) that CRF rapidly induces c-*fos* expression by cAMP- and Ca^{2+}-dependent mechanisms; and 2) that overexpression of cFOS increases POMC gene expression

RESULTS

CRF Rapidly Induces c-*fos* in Pituitary Cells

To determine whether c-*fos* induction is involved in the early response to CRF, c-*fos* mRNA was analyzed in AtT20 cells after exposure to CRF (10^{-8} M). Northern blots were prepared in duplicate and used to measure c-*fos* or POMC mRNA. The c-*fos* transcripts increased 15-fold after 60 min of CRF treatment and then declined to initial levels within 4 h (Fig. 1). POMC mRNA levels followed a different time course, since increased levels were observed only after 4 h of CRF treatment (Fig. 1), in agreement with previously reported data (5)

Secretagogues Acting on cAMP and Calcium Increase c-*fos* Levels in AtT20 Cells

The effects of several drugs that stimulate POMC-derived peptide secretion from AtT20 cells were compared to the effect of the CRF stimulus on c-*fos* mRNA accumulation (Fig. 2). Cells were treated for 1 h, and

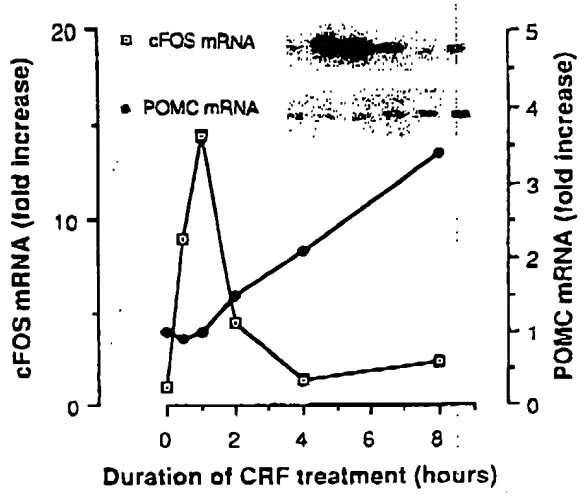

Fig. 1. CRF Induces c-*fos* and POMC mRNA in ATt20 Cells

Cells were grown to 50% confluency and serum starved for 24 h before treatment. The c-*fos* and POMC mRNA levels were measured by Northern blot, and 5 μg were loaded in each lane. CRF (10 nM) was added progressively, 8, 4, 2, 1, and 0.5 h before harvesting. The *inset* shows typical autoradiograms. Measurments of relative c-*fos* and POMC mRNA levels were performed by scanning the autoradiograms with a BIOCOM 200 image analyzer.

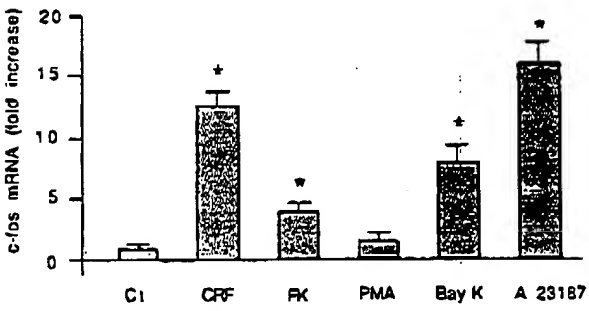

Fig. 2. C-*fos* Is Induced by CRF, FK, and Ca^{2+} Entry

Cells were grown to 50% confluency, then serum starved for 24 h before a 60-min treatment with CRF (10^{-8} M), FK (5×10^{-6} M), PMA (10^{-5} M), Bay K 8644 (10^{-7} M), or the Ca^{2+} ionophore A 23187 (1 mM). Total mRNA was extracted and subjected to dot blot analysis. Serial dilutions of each sample corresponding to 2.5, 1.75, and 0.87 μg total RNA were applied to the filters. Data represent the mean ± SEM of at least six individual experiments. The *asterisk* indicates $P \leq 0.05$ when compared to untreated control cells (by Student's *t* test).

mRNA levels were quantified by mRNA dot blot analysis. CRF (10^{-8} M) treatment resulted in a 12-fold increase in c-*fos* mRNA levels, a result comparable to that obtained by Northern blot analysis. Direct activation of adenylate cyclase with forskolin (FK; 5×10^{-6} M) increased c-*fos* levels 4-fold. The role of Ca^{2+} was investigated by increasing intracellular free Ca^{2+} concentrations. Treatment with the Ca^{2+} ionophore A 23187 (10^{-6} M) or the Ca^{2+} channel agonist BAY K 8644 (10^{-7} M) increased c-*fos* mRNA levels to extents comparable to those observed with CRF. Cotreatment

with the ionophore and FK did not further increase the c-*fos* induction. Treatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA; 10^{-8} M for 1 h; Fig. 2) was without significant effect either when applied alone (10^{-10}-10^{-7} M for 1 h and 10^{-8} M at different time points up to 8 h) or in combination with FK (data not shown)

Induction of c-*fos* by CRF Requires a Functional Calcium/Calmodulin (CAM) Pathway

We addressed the ability of Ca^{2+} to mediate the second messenger-mediated effects of CRF induction in AtT20 by blocking Ca^{2+} entry with antagonists during a 1-h CRF challenge. Cells were pretreated with D600 (10^{-5} M) and nifedipine (10^{-7} M) for 10 min, followed by CRF treatment. These Ca^{2+} channel blockers decreased the CRF-induced c-*fos* signal by 60% relative to that in controls. Similarly, removal of extracellular Ca^{2+} completely abolished the c-*fos* induction. Pretreatment with either CAM inhibitor W_7 or W_{13} (both at 10^{-7} M) decreased c-*fos* induction by 50–60% (Fig. 3). These treatments (D600, nifedipine, and W7) did not significantly alter basal or CRF- or FK-stimulated cAMP levels, as previously reported by Miyazaki and co-workers (6)

Induction of c-*fos* by CRF Is Dependent on PKA

Due to the lack of selectivity of classical protein kinase inhibitors, we chose to examine the role of PKA by overexpressing either the catalytic subunit of PKA or different forms of the regulatory PKA using a vector (Mt-REV_{AB}) expressing a point-mutated regulatory subunit to which cAMP is unable to bind. In this latter case, overexpression of this mutated subunit should down-titrate the endogenous catalytic subunits and inactivate the PKA complex. McKnight and colleagues (23) and others (24) have demonstrated that such a manipulation can efficiently block the PKA-mediated stimulation of cAMP-responsive genes. The efficiency of the method was verified in our experimental model by cotransfecting the expression vectors with a plasmid containing a canonical cAMP-responsive element (CRE) linked to a chloramphenicol acetyl transferase (CAT) reporter gene (CRE/tK-CAT). Figure 4A shows that this construct responds to direct activation of adenylate cyclase by forskolin ($5 \cdot 10^{-6}$ M) in AtT20 cells. This response to forskolin is completely lost when the cells are cotransfected with Mt-REV_{AB} (see Fig. 4A) coding for the mutated regulatory subunit of PKA. In cells in which PKA was thus inactivated (Fig. 4B), the rise in c-*fos* mRNA induced by CRF (10^{-8} M) was strongly reduced (80–90%) compared to that in control cells. Expression of this mutated form of the regulatory subunit did not alter the rise in cAMP levels after CRF treatment (not shown).

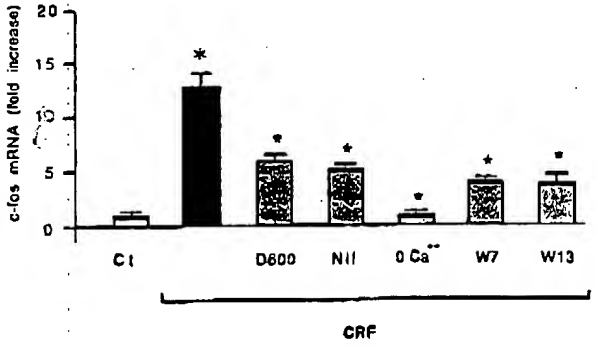

Fig. 3. Induction of c-*fos* by CRF Is Inhibited by Ca^{2+} Antagonists and CAM Inhibitors

CRF (10^{-8} M) was applied for 1 h. Ten minutes before this stimulation, D600 (10^{-5} M), nifedipine (Nif; 10^{-7} M), Ca^{2+}-free DMEM (0 Ca^{2+}), or W7 and W17 (10^{-7} M) were given. Total RNA was analyzed by dot blot, as shown in Fig. 2. Data represent the mean ± SEM of at least six individual cultures. The *asterisk* indicates $P \leq 0.05$ (by Student's *t* test): * when compared to untreated controls and ★ when compared to CRF-stimulated cells.

The role of the cAMP pathway in the regulation of c-*fos* gene expression in AtT20 cells was further investigated by using reporter plasmids containing either 404 basepairs (plasmid FC4) or 220 basepairs (plasmid FC8) of the 5′ flanking region of the human c-*fos* promoter coupled to the CAT reporter gene (25) (Fig. 5A). The contribution of PKA to upregulate c-*fos* transcription was evaluated by overexpressing the catalytic subunit of this enzyme. Cotransfection of FC4, FC8, or CRE/tK-CAT with an expression vector coding for the catalytic subunit of PKA strongly increased CAT activity (Fig. 5B). Figure 5C shows that these c-*fos* promoter constructs, like the endogenous c-*fos* gene, are induced 9- and 6-fold by CRF and FK, respectively. The c-*fos* promoter constructs smaller than 60 basepairs or a tK-CAT vector without CRE were not stimulated by CRF or cotransfection with the catalytic subunit.

Our next step was to demonstrate whether c-*fos* induction by CRF was dependent on a functional PKA pathway. Again, cotransfecting FC4 along with Mt-REV_{AB} almost completely abolished the stimulatory effect of CRF on c-*fos* transcription (Fig. 6B), whereas cotransfecting a control plasmid (Fig. 6A) or a plasmid expressing the wild-type regulatory subunit (Fig. 6C) did not inhibit these responses. Transcription from a construct bearing the simian virus-40 long terminal repeat (SV40 LTR) linked to the CAT sequence was not modified by coexpression of the mutated regulatory subunit.

Role of PKA in Control of the cAMP and Calcium Pathway in AtT20 Cells

To determine at which level of the intracellular second messenger cascade PKA interacts with the Ca^{2+} pathway, we introduced the FC4 reporter plasmid into AtT20 cells with either control plasmids (pCH 110 or pUC 18) or Mt-REV_{AB}. In the former case, CRF (10^{-8} M), FK ($5 \cdot 10^{-6}$ M), K^+ (20 mM), and BAY K 8644 (10^{-7} M) each stimulated c-*fos*-directed CAT activity 9-, 6-, 5-, and 4.5-fold, respectively, in three to five independent

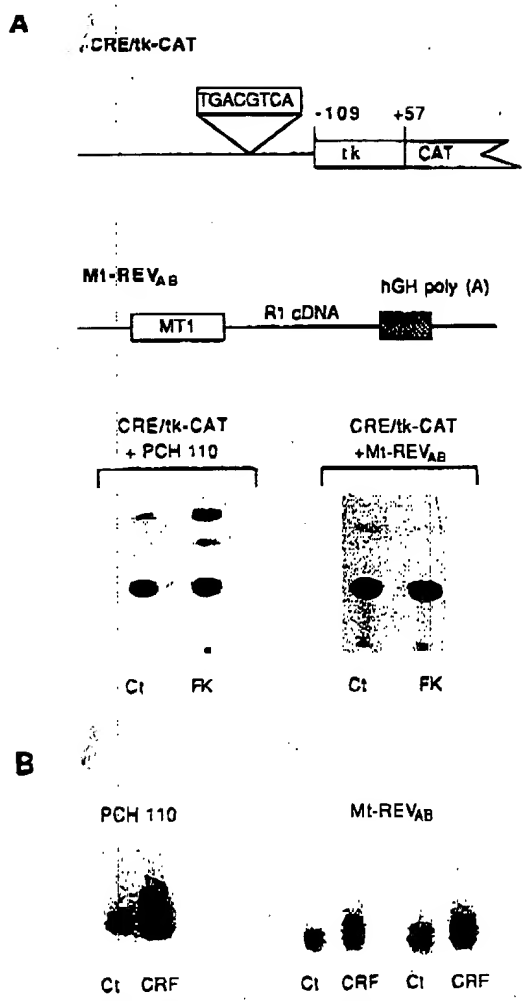

Fig. 4. Mutated Regulatory PKA Subunits Abolish cAMP-Dependent Induction of CRE-Containing Genes

A, AtT20 cells were cotransfected (see *Materials and Methods*) with a CRE-containing gene, CRE/tK-CAT (1 μg/well), with either an expression vector (2 μg/well) coding for a mutated regulatory PKA subunit lacking cAMP-binding sites (Mt-REV$_{AB}$) or with a control plasmid (2 μg/well; PCH110, an expression vector coding for β-galactosidase, or pUC18). The transfection step lasted 10 h, after which cells were serum deprived for 24 h and then stimulated with FK (5×10^{-6} M) for 10 h. CAT activity was determined and taken as an index of CRE/tK-CAT transcription. Induction of CRE/tK-CAT transcription by FK was completely abolished in AtT20 cells cotransfected with Mt-REV$_{AB}$, contrary to that in cells cotransfected with either PCH110 or pUC18. B, After 12 h of serum deprivation in DMEM, AtT20 cells were transfected with Mt-REV$_{AB}$ (2 μg/well; 10 h). After an additional 24-h period in serum-free DMEM, cells were stimulated with CRF (10^{-6} M) for 1 h. Total RNA was extracted and analyzed by Northern blot (5 μg/lane). Expression of the mutated regulatory subunit of PKA resulted in a marked decrease in c-*fos* mRNA accumulation after CRF treatment compared with that in cells transfected with PCH110. The experiment was repeated three times in duplicate with similar results.

experiments (Fig. 6A). In cells transfected with Mt-REV$_{AB}$, K^+ and BAY K 8644 continued to induce c-*fos* expression, even through in the same experiment CRF or FK inducibility was abolished (Fig. 6B). Overexpression of the wild-type form of the regulatory subunit Mt-R$_{WT}$ (Fig. 6C) did not inhibit c-*fos* transcription. This control experiment shows that the inhibition observed upon transfection with a plasmid bearing the mutated sequence does not result from unspecific promoter effects.

cFOS Stimulates POMC Transcription

The physiological meaning of c-*fos* induction after CRF treatment was further investigated by addressing the question of whether cFOS modulates POMC transcription. In a genetic approach, we used the POMC promoter (700 basepairs; see Fig. 7A) linked to the CAT sequence pJL145 (26). This sequence has been previously shown to contain the regulatory element(s) responsible for tissue-specific expression (27) of this gene. Cotransfection of this construct with an expression vector coding for the human cFOS protein pBK28 (28) stimulated POMC transcription 5-fold in more than 10 independent experiments. The effect of pBK28 on POMC expression was compared to that in control cells cotransfected with either an unrelated plasmid (PCH110) or a frame shift mutant of pBK28. Two typical and independent experiments are shown in Fig. 7B. This latter experiment, thus, shows that transactivation of the POMC promoter is dependent on functinal *cFOS* protein and rules out unspecific promoter effects (titration of regulatory proteins, for example). In parallel experiments, a promoterless but otherwise identical CAT construct was not stimulated by the *cFOS* expression vector. Furthermore, stimulation of the POMC gene does not reflect a general increase in transcription induced by overexpression of *cFOS*, since basal and serum-induced transcription from the c-*fos* promoter (construct FC4) were efficiently inhibited in this cotransfection assay, as previously described in other cell types (29).

DISCUSSION

The neurohormone CRF stimulates POMC gene expression and secretion of POMC-derived peptides in pituitary corticotrophs and melanotrophs as well as in the corticotroph-derived AtT20 cell line (2–4, 8, 10, 11). In the present report we analyzed the effects of CRF on the induction of the protooncogene c-*fos* in this cell line by direct measurement of c-*fos* mRNA levels or by gene transfer experiments. We show that in these cells the CRF-induced increase in POMC mRNA levels is temporally preceded by an increase in the level of transcripts coding for c-*fos* mRNA. The c-*fos* response is rapid (detectable within 30 min of CRF stimulation) and transient (Fig. 1), in support of the self-limiting

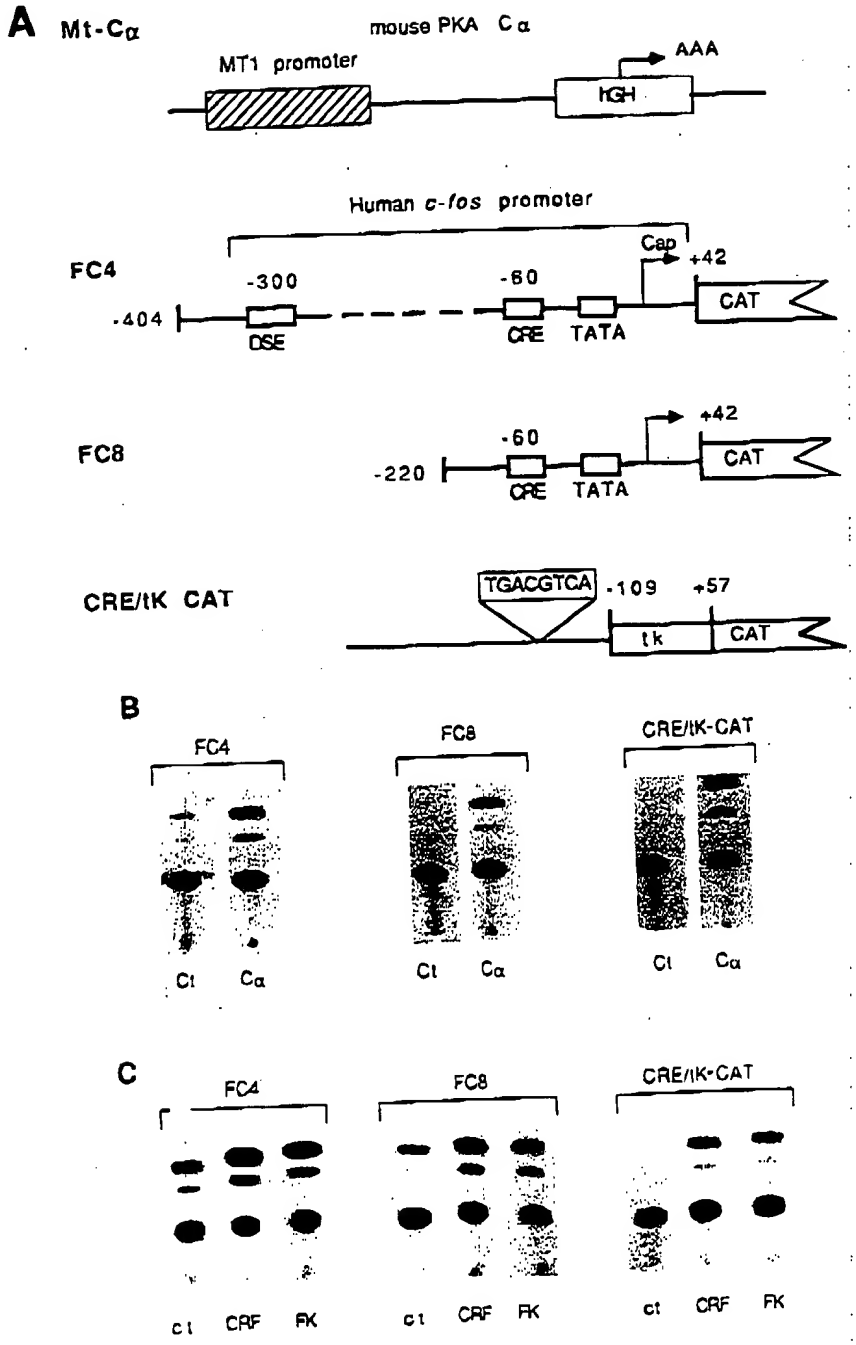

Fig. 5. *c-fos* Transcription Is Stimulated by cAMP and PKA

A, The direct contribution of PKA in the induction of c-*fos* was analyzed by overexpressing the catalytic subunit of PKA vector Mt-Cα in combination with CAT reporter genes bearing 5′ flanking sequences of the human c-*fos* promoter vectors FC4 and FC8; some characterized regulatory elements are indicated by *boxes* or a CRE. B, After the same treatment paradigm as that described in Fig. 4B, cells at 30–50% confluency were serum deprived for 12 h and transfected for 10 h (2 μg reported genes and 1 μg Mt-Cα or PCH110/pUC18), and after an additional 24-h period in serum-free DMEM, CAT activity was determined. Overexpression of a catalytic subunit of PKA in AtT20 cells increases transcription of the CAT reporter gene containing sequences of the c-*fos* promoter FC4, FC8, or a CRE (CRE/tK-CAT). Similar results were obtained in three independent experiments. C, Chimera genes bearing −404 FC4 or −220 FC8 basepairs of the human c-*fos* promoter region or a CRE (CRE/tK-CAT) linked to the CAT-coding sequence are inducible by CRF (10^{-8} M) and FK (5×10^{-6} M). Cells were transfected with 2 μg DNA for 10 h. After 24 h in serum-free medium, drugs were added for 12 h, and CAT activity was determined.

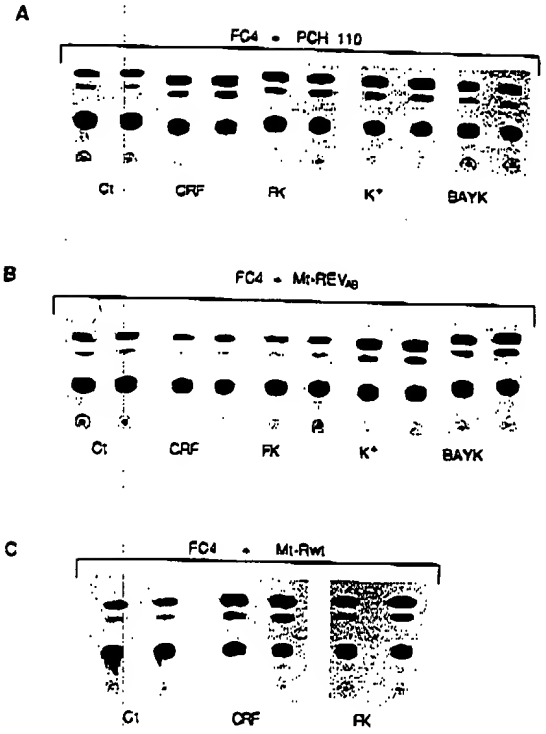

Fig. 6. Inhibition of PKA Selectively Suppresses cAMP-Regulated *c-fos* Induction in AtT20 Cells

After 12 h of serum deprivation, cells were transfected with 2 μg FC4 in combination with 2 μg PCH110 or pUC18 (A), Mt-REV_{AB} coding for the mutated regulatory subunit of PKA (B), or Mt-R_{WT} coding for the normal regulatory subunit. Twenty-four hours after the transfection step, cells were treated for 12 h with CRF (10^{-8} M), FK (5×10^{-6} M), K^+ (25 mM), or the Ca^{2+} channel agonist BAY K 8644 (10^{-7} M). A, Each stimulation resulted in increased CAT activity in cells cotransfected with PCH110 or pUC18). B, Expression of the regulatory subunit that lacks cAMP-binding sites suppresses CRF and FK stimulation, whereas K^+- and BAY K-stimulated CAT production was unchanged. C, Expression of the wild-type form of the regulatory subunit failed to block the CRF- or FK-induced increase in CAT activity.

genomic shut-off process well documented for this protooncogene (29). The transient nature of the CRF-induced *c-fos* mRNA signal does not result from receptor down-regulation, since *c-fos* mRNA accumulation after cytosolic cAMP and Ca^{2+} elevation with FK or ionophore A 23187 treatment follows a biphasic time course similar to that generated by CRF (data not shown).

Analysis of CRF Second Messengers

We used direct measurements of *c-fos* mRNA and gene transfer experiments to analyze the second messenger systems relaying the CRF signal in AtT20 cells. Since cAMP and intracellular calcium ion concentrations are modulated by CRF, cells were treated with agents that directly affect these two regulatory pathways. Activation of adenylate cyclase with FK (5×10^{-6} M) increased *c-fos* mRNA levels. However, this increase (4-fold) was smaller than the 12-fold increase observed with CRF (Fig. 2). Larger doses of FK (up to 25 μM) have been tested, yet no greater inductional effects were observed. These observations might indicate that CRF directly activates another regulatory pathway which cooperatively interacts with the cAMP second messenger system. This second pathway may be independent of cAMP elevation and PKA activation, since CRF (10^{-8} M) and FK (5×10^{-6} M) produced a comparable increase in cAMP levels (data not shown). This secondary regulatory branch could be triggered by changes in the intracellular Ca^{2+} concentrations arising from extracellular sources or mobilization of intracellular stores. Mobilization of Ca^{2+} from intracellular stores is thought to result from production of inositol phosphates by phospholipase-C activation, which also results in activation of PKC (30). Although the phorbol ester PMA activates PKC and is a strong inducer of *c-fos* in many cell types (31–34), PMA did not modify *c-fos* mRNA levels in our experiments in AtT20 cells at the various concentrations and treatment times tested. It has been recently suggested that *cFOS* is a specific phosphorylated target for the β1 PKC subtype (35), one of several PKC subtypes. Therefore, the lack of any detectable *c-fos* response to PMA could reflect the absence of PKC β1 in these pituitary cells, suggesting that CRF does not stimulate *c-fos* via PKC in AtT20 cells.

In contrast to the effects of PKC activation, the use of drugs that elevate intracellular free calcium ions by increased entry of extracellular Ca^{2+} stimulates *c-fos* mRNA levels (Fig. 2) and transcription from the *c-fos* promoter (Figs. 5 and 6). Treatment with the dihydropyridine BAY K 8644 increased *c-fos* mRNA levels 8-fold relative to control values, whereas a more direct manipulation of Ca^{2+} entry with the Ca^{2+} ionophore A 23187 produced a 15-fold increase, comparable to that observed with CRF. Furthermore, the organic blockers of Ca^{2+} channels, nifedipine and D600, decrease CRF induction of *c-fos* mRNA by 60%, and removal of Ca^{2+} ions from the extracellular medium completely abolishes the CRF-mediated effect on *c-fos* mRNA levels (Fig. 3).

Cotreating AtT20 cells with the CAM antagonists W7 and W13 also reduced the *c-fos* mRNA signal. These data suggest that the intracellular free Ca^{2+} increase generated by CRF leading to *c-fos* stimulation is mediated by CAM and probably relayed by a Ca^{2+}/CAM-dependent protein kinase. The conclusion, that this enzyme can regulate the *c-fos* protooncogene, would be in line with that reached by Morgan and Curran (14) from early studies in PC 12 cells. Thusfar, our results implicate that CRF strongly induces *c-fos* mRNA by increased intracellular Ca^{2+} ion concentrations arising from extracellular sources and activation of CAM-dependent kinases. In addition, since CRF activates adenylate cyclase, and FK also induces *c-fos* mRNA (Fig. 2) in these cells, the CRF-mediated *c-fos* response may involve cross-talk between cAMP- and Ca^{2+}-dependent systems.

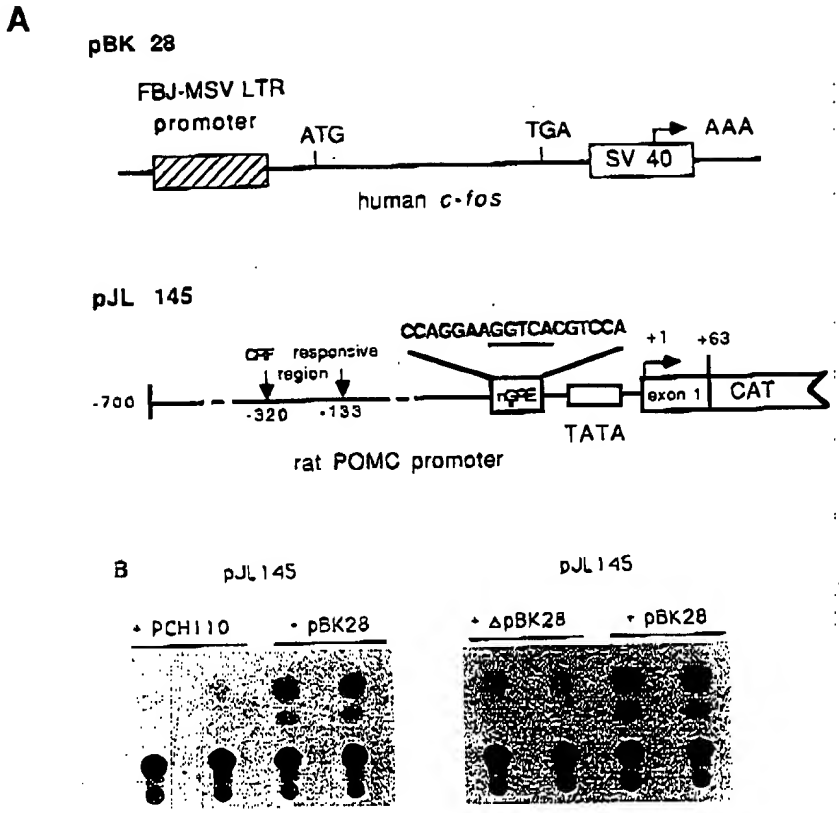

Fig. 7. The *cFOS* Transactivates POMC in Corticotrope Cells

A, AtT20 cells were cotransfected with a vector containing 700 basepairs of the rat POMC promoter coupled to the CAT sequence pJL145 (2 μg/well) and an expression vector pBK28 coding for the human *cFOS* (2 μg/well). Regulatory regions of the POMC promoter by the two main physiological modulators are depicted: the CRF-responsive region (26) and the negative glucocorticoid receptor element (nGRE) (42). Note within the negative glucocorticoid-responsive sequence, homology to the half-palindromic estrogen receptor is *underlined*. Controls received 2 μg vector PCH110 or ΔpBK28, a frame shift mutant of pBK28. The transfection step lasted 12 h, and after an additional 24-h period in DMEM, CAT activity was determined. B, An autoradiogram from two independent experiments.

Genetic Analysis to Study the Role of PKA in c-*fos* Induction

To analyze the role of PKA in transmiting intracellular signals generated by CRF, cloned cDNA sequences coding for individual subunits of PKA were transiently expressed in AtT20 cells. In an elegant series of experiments, McKnight and co-workers (23, 24) previously demonstrated that overexpressing the catalytic subunit of the α-isoform of the mouse PKA can directly activate gene transcription via cAMP-responsive DNA elements.

Although these experiments were primarily designed to study mechanisms by which CRF induces c-*fos* in pituitary cells and not to analyze specific genomic CRF-responsive elements within the c-*fos* promoter, different constructs were used (Fig. 5). Our results indicate that overexpression of the catalytic subunit of PKA (Cα) stimulates CAT transcription from a construct (FC4) containing the major regulatory sequences described thusfar, including the Dyad symetry element (DSE), multiple CRE, and a putative AP1 site. This latter is probably not relevant to c-*fos* induction by CRF, since TPA does not stimulate endogenous c-*fos* mRNA (Fig. 2) or transcription from FC4 (not shown). Furthermore, the DSE does not appear to be required for CRF induction of c-*fos*, since FC8, which lacks this sequence, is efficiently stimulated by CRF and Cα. The CRE (TGACGTCA) was then tested in a heterologous promoter construct and found to respond to CRF and Cα. This does not exclude additional CRF-responsive sequences. Clearly, multiple sequences within the c-*fos* promoter respond to cAMP (18). Furthermore, Greenberg's group (19) recently reported that deletion of the c-*fos* CRE (or Ca^{2+}-responsive element) located at −60 basepairs did not modify the induction of an otherwise intact c-*fos* promoter. Taken together, our data indicate that a CRE is at least one genomic target for c-*fos* induction by CRF. The catalytic subunit of PKA may be translocated into the nucleus (36), activating transcription as a result of direct phosphorylation of transacting factors such as cAMP-responsive element-binding protein (19, 37, 38).

We next applied gene transfer techniques to determine whether CRF stimulation of c-*fos* involved PKA-dependent modulation of Ca^{2+} entry in AtT20 cells. Here we expressed a mutant form of the regulatory

subunit of PKA by transfecting cells with Mt-REV$_{AB}$. This mutated subunit does not bind cAMP and, therefore, blocks PKA activation. When sufficiently expressed, it titrates down endogenous catalytic subunits and inhibits the PKA pathway (23, 24). When cells were cotransfected with Mt-REV$_{AB}$ and CRE/tk-CAT, FK induction of CAT activity was completely abolished as a result of inhibited PKA activity (Fig. 4A). In AtT20 cells, this treatment resulted in a strong (~80%) reduction in c-*fos* mRNA stimulation by CRF (Fig. 4B). It is unclear whether the residual increase with CRF treatment is independent of PKA or is due to a signal observed in untransfected cells requiring further experiments using stably transfected AtT20 cell lines expressing the mutated regulatory subunit. We then examined whether blocking PKA hindered c-*fos* induction by CRF or treatments increasing cAMP or Ca^{2+} entry. As seen in Fig. 6, in cells cotransfected with a c-*fos* promoter-CAT construct (FC4) and Mt-REV$_{AB}$, CRF and FK induction of c-*fos* transcription is eliminated, whereas in the same experiment, it continues to be induced by agents inducing Ca^{2+} entry, such as K^+ or BAY K 8644, suggesting that the Ca^{2+}/CAM system could modulate c-*fos* independently of PKA. This set of experiments shows that c-*fos* induction by CRF is dependent on a functional PKA system. Thus, the Ca^{2+} component of the CRF stimulus may result from an action of the PKA at the level of the cell membrane, possibly by phosphorylation of Ca^{2+} channels (39) and subsequent increased Ca^{2+} flow into the cell. This interpretation is consistent with the observation that CRF increases Ca^{2+} entry in AtT20 cells secondary to a rise in intracellular cAMP levels (7).

However, the mechanism by which CRF increases gene transcription is probably more complex. In addition to its direct coupling to the adenylate cyclase, the CRF receptor may be directly coupled to other effector systems, such as phospholipase-A or -C. Such an additional effector system is suggested by our observation that FK does not produce a massive stimulation of c-*fos*, although cAMP is increased to comparable levels when cells are challenged with FK and CRF. Clearly, since inactivation of PKA completely suppresses c-*fos* induction, PKA would play a permissive role, perhaps allowing coupling of the CRF receptor to other effector systems.

Functional Relevance of c-*fos* Induction by CRF

The contribution of immediate early genes is not restricted to cell cycle-related events, but the genes products may also act as intracellular messengers in cells that have stopped their mitotic program (*e.g.* neurons and endocrine cells). This would agree with our observation that CRF increases c-*fos* expression in primary (nondividing) melanotroph or anterior pituitary cultures (Loeffler, J. P., and A. L. Boutillier, unpublished observation). Thus, the c-*fos* protooncogene may also represent a transduction mechanism for regulating other pituitary genes controlled by CRF. We have analyzed its effect on the main hormonal product of corticotrope cells, POMC. As shown in Fig. 7, POMC transcription is efficiently stimulated by c*FOS* in a cotransfection assay. Whether this results from a direct transactivation of POMC promoter or implicates a cascade of other genes remains to be established. It has recently been shown that glucocorticoid receptor (GR) activity is potently inhibited by c*FOS* and c*JUN*. This mechanism appears to involve direct protein/protein interaction between GR and either c*FOS* or c*JUN*. This effect is independent of DNA binding (40, 41). The POMC promoter is negatively regulated by glucocorticoids (1, 42). If one speculates that the GR represses basal POMC gene activity, the observed increase in POMC transcription upon cotransfection with the c*FOS* expression vector could result from down-titration of functional GR. In this case, the c*FOS* protein would not necessarily interact physicaly with POMC promoter sequences. However, the inhibitory glucocorticoid-responsive sequence located at −60 basepairs in the POMC promoter (42) also overlaps a sequence homologous to the half-palindromic estrogen-responsive element (5′-GGTCA-3′; see Fig. 7A). This sequence can mediate phorbol ester induction and bind the *FOS/JUN* complex (43). Since c*JUN* is expressed at high levels in AtT20 cells (Loeffler, J. P., unpublished observation), this sequence may represent a regulatory element modulated by the AP1 complex. We are currently examining the POMC promoter by site-directed mutagenesis and footprinting analysis in order to help clarify this point.

In summary, our study demonstrates that CRF induces c-*fos* in corticotrope cells. This induction is dependent upon an increase in cytosolic cAMP levels and flux of Ca^{2+} of extracellular origin, and the signals generated by these second messengers may ultimately be transduced by the transacting factor CRE-binding protein. c*FOS* activates the POMC promoter, and stimulation of c-*fos* by CRF may participate in the delayed induction of POMC gene expression.

MATERIALS AND METHODS

Cell Culture

AtT20/D-16V mouse pituitary tumor cells (a generous gift from J.L. Roberts, New York, NY) were propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, glutamine (286 mg/liter), penicillin (100 μg/ml), streptomycin (100 μg/ml), and kanamycin (50 μg/ml). Cells were plated on Costar dishes (Cambridge, MA; diameter 3 cm) and cultured at 37 C under 95% O_2-5% CO_2 until 30–50% confluency was reached. Cells were then serum starved (no fetal calf serum, same medium as above) for 24 h before experiments or transfection studies.

Isolation of mRNA and Hybridization Studies

After stimulation with the appropriate drugs, total RNA was extracted by the LiCl method, according to Auffray and Rougeon (44). RNA was quantitated by UV absorption (1 A_{260} = 40 μg RNA/ml), and 5 μg total RNA were used for Northern or dot blot analysis, as previously described (45). After transfer, the nitrocellulose filters were prehybridized (5 h) and hybridized

(16 h; 5000 cpm ^{32}P-labeled probe/cm^2) according to the method of Wahl *et al.* (46). Thereafter, filters were washed, dried, and autoradiographed at −70 C. The autoradiograms were quantitated with an LKB Ultrascan densitometer (Rockville, MD) or a Biocom 200 Image analyzer (Biocom, France).

Plasmids and Probes

POMC mRNA was quantified, as previously reported (45), with a single stranded M13 probe complementary to the mouse β-endorphin-coding sequence (obtained from Dr. J. L. Roberts, New York, NY).

The *c-fos* mRNA was measured with a ^{32}P random prime labeled fragment (kit from Boehringer, Mannheim, Germany) complementary for 470 basepairs of the rat *c-fos* gene (a generous gift from Dr. R. Muller, Heidelberg, Germany).

Reporter Gene and Expression Vectors

The somatostatin CRE oligodeoxynucleotide (core sequence TGACGTCA), which covers the −32/−61 promoter region and is linked to the thymidine kinase (tK) promoter from the herpes simplex virus, generates CRE/tK-CAT (see Fig. 4) (37).

Recombinants FC4 (−404) and FC8 (−220; see Fig. 5) containing the progressively deleted human *c-fos* promoter linked to CAT sequence have been described previously (25). Plasmid Mt-Cα (see Fig. 5) expresses the catalytic subunits of the mouse PKA, and Mt-REV$_{AB}$ (see Fig. 4) expresses a mutant form (without cAMP-binding sites) of the regulatory subunit of type I PKA. Plasmid Mt-R$_{WT}$, which served as a control, expresses the wild-type regulatory subunit. These constructs (a generous gift from Dr. Stanley McKnight, Seattle, WA) have been described previously (23, 24). Since basal expresion from the metallothionein promoter was sufficient for our purposes, Zn^{2+} or Cd^{2+} treatment (which severely interferes with Ca^{2+} metabolism) was avoided. The vector expressing human cFOS, pBK28 (28), was a gift from I. Verma (San Diego, CA), and pJL145 (26) containing the rat POMC promoter was generously provided by J. L. Roberts (New York, NY).

PCH 110 and pUC 18 were used to keep constant the amount of total DNA in cotransfection experiments and were obtained from Pharmacia (Freiburg, Germany).

Transfections and CAT Measurement

Transfection of AtT20 cells (~50% confluency) was carried out with a lipopolyamine-based method previously described in detail (47, 48). Briefly, DNA (2 or 5 μg/well) was mixed with 4 or 10 μl of a 2 mM aqueous solution of dioctadecylamidoglycylspermine in 1 ml DMEM. Under these conditions, DNA is efficiently coated with a lipid layer and enters the cells. The transfectant solution was applied overnight to cells grown in Costar (3-cm) culture dishes. After that period, cells were rinsed and grown in DMEM culture medium.

CAT activity was determined by the method of Gorman *et al.* (49). Cells were suspended in 100 μl 200 mM Tris-HCl (pH 7.4). After several freeze/thaw cycles, the extract was heated (65 C) for 10 min and centrifuged (14,000 × *g*; 5 min), and 80 μl of the supernatant were added to 40 μl Tris-HCl containing [^{14}C]chloramphenicol (0.1 mCi). After 5 mn at 37 C, the reaction was initiated by adding 40 μl 4 mM acetyl coenzyme-A, incubated for 2 h at 37 C, and extracted with 0.5 ml ethyl acetate. After analysis by TLC, the acetylated and unreacted forms of chloramphenicol were located, cut out, and counted. CAT activity was calculated as the percentage of chloramphenicol converted to acetylated forms. It was found that the transfection efficiency, at a constant DNA concentration, varied less than 15% within a given experiment. Therefore, the use of internal controls, such as β-galactosidase expression vectors, to account for differences in transfection efficiency, as often used with other methods, was unnecessary. The total protein content in each reaction was measured by the method of Bradford (kit from Bio-Rad, Paris, France) and varied less than 10% between the different extracts.

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Thank you

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Down-regulation of the protein kinase A pathway by activators of protein kinase C and intracellular Ca^{2+} in fibroblast cells

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Abstract Many genes are regulated by the intracellular calcium, protein kinase C (PKC) and protein kinase A (PKA) pathways and it has been shown that these pathways synergize in some cell types, whereas they antagonize in others. Here we show that the calcium and PKC pathways suppress the effects mediated by the PKA pathway in a fibroblast cell line. The suppressing effect of elevated intracellular Ca^{2+} levels, but not of the PKC pathway, can be abrogated by the addition of cyclosporin A (CsA), indicating that the effect of Ca^{2+} is mediated by phosphatase-2B (PP-2B/calcineurin). Suppression by the PKC pathway is not mediated by the proto-oncogenes c-*fos*, c-*jun* and *junB*, as the co-transfection of these genes does not block the effects of the PKA stimulator 8-Br-cAMP. In addition, cotransfection with the catalytic subunit of PKA shows that the inhibitory effect of PKC occurs upstream of PKA activation.

Key words: Signal transduction; Gene regulation; Transcription factor; Cross-talk of signalling pathways

1. Introduction

The cell receives many signals from outside through different signalling pathways. Among the most studied pathways are the protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+} pathways. The PKC and Ca^{2+} pathways nearly always synergize, as they are often triggered simultaneously by a membrane-bound receptor, whereas depending on the cell type, they sometimes antagonize or synergize with the PKA pathway [1].

The individual signalling pathways have been studied intensively; however, less work has been devoted to elucidate the mechanisms of the interactions among the different signalling pathways. To study the antagonism of the PKA, PKC and Ca^{2+} pathways in mouse fibroblasts we used defined reporter plasmids containing cAMP responsive elements (CRE) or TPA responsive elements (TRE) in front of the chloramphenicol acetyl transferase (*CAT*) reporter gene. The CREs are targets of the transcription factor CREB [2] which is activated through the PKA and Ca^{2+}/calmodulin pathways, whereas the TREs are targets of the products of the *fos* and *jun* gene families which are activated by the PKC pathway [3] and elevated levels of intracellular Ca^{2+} [4]. The results we obtained with this system show that the PKC and Ca^{2+} pathways dominate over the PKA pathway in mouse fibroblasts when they are stimulated at the same time.

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2. Material and methods

2.1. Cell culture and transient DNA transfection

L4 cells [5] which are derivatives of the mouse fibroblast cell line NIH 3T3 were grown in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% new-born calf serum (NBCS), 2.5 μg/ml amphotericin B, 5 mg/ml gentamicin, and 2 mM glutamine and were transfected by calcium phosphate co-precipitation as described in [6]

2.2. Expression vectors

The expression vector pCEV contains the catalytic subunit (c alpha) of the mouse PKA [7], whose expression is driven by the metal ion-inducible mouse metallothionein promoter. The expression of c-*fos*, c-*jun*, and *junB* was driven from the RSV (Rous sarcoma virus) enhancer of their vectors RSV-c-fos [8], RSV-c-jun [9], and RSV-junB [10]. Expression of the beta-galactosidase reference gene was driven from the CMV (cytomegalovirus) enhancer of the expression vector pCMV-beta-gal.

2.3. CAT assay

Chloramphenicol acetyl transferase (CAT) assays were performed mainly following the protocol of Gorman et al. [11] with the exception that the whole-cell extracts were heated to 65°C for 10 min to destroy deacylases and that the incubation time was extended to 16 h. As reporter genes we used 2×*SOM-CRE-CAT* [12] containing two CREs of the *somatostatin* gene [2] in front of a TATA-box and the *CAT* gene; and 5×*TRE-CAT* [13] in which five TPEs [3] have been inserted in front of a TATA-box and the *CAT* gene. For each experiment three independent transfections were carried out and each whole-cell extract obtained was tested twice for CAT activity. From the resulting six measurements the average value and standard deviation were obtained. The CAT assays were standardized by cotransfecting 0.1 μg of the expression vector pCMV-beta-gal by and measuring the beta-galactosidase activity of whole-cell extracts prepared from one-quarter of the transfected cells.

3. Results

3.1. Stimulation of gene transcription by the PKA, PKC and Ca^{2+} signalling pathways in L4 cells

We first studied the effect of 8-Br-cAMP (1 mM) on the reporter plasmid 2×*SOM-CRE-CAT* [12] in the absence and presence of thapsigargin (10 nM) a mobilizer of intracellular calcium. Fig. 1 shows that 8-Br-cAMP stimulates CAT from the 2×*SOM-CRE-CAT* plasmid approximately 5-fold. This stimulation is totally reversed when 8-Br-cAMP and thapsigargin are given simultaneously. The same effects are observed when instead of the 8-Br-cAMP treatment the catalytic subunit of PKA was co-transfected. The addition of cyclosporin A (CsA, 10 ng/ml), an inhibitor of the calcium-dependent phosphatase PP-2B (calcineurin) [14] totally relieved the suppressing effect of thapsigargin, indicating that the effect of thapsigargin is mediated by this phosphatase. The same effects are observed when instead of 8-Br-cAMP treatment the catalytic subunit of PKA is transfected into L4 fibroblasts, i.e., the approximate 6-fold stimulation by the PKA catalytic subunit is suppressed by thapsigargin and this suppression is relieved

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by CsA. These experiments indicate that the dominance of the Ca^{2+} pathway over the PKA pathway is mediated by PP-2B.

We next tested whether the PKC activator TPA had an effect on 8-Br-cAMP and PKA catalytic subunit-stimulated CAT expression from the 2×*SOM-CRE-CAT* reporter plasmid. Fig. 2 shows that the 8-Br-cAMP-stimulated *CAT* gene expression from this plasmid is totally blocked by TPA. In contrast to thapsigargin this suppression is not relieved by CsA. This result indicates that suppression of the PKA pathway by TPA is not mediated by PP-2B.

To see whether the effect of TPA is mediated by the transcription factor AP-1, we transfected expression vectors for the c-*fos* and c-*jun* gene together with the 2×*SOM-CRE-CAT* reporter plasmid into L4 fibroblast cells and treated them with mM 8-Br-cAMP. The AP-1 transcription factor is a heterodimer of the gene products of members of the *jun* and *fos* gene families and is activated by TPA. AP-1 binds to a DNA sequence which is very similar to that of CREB and it may be possible that the competition for the CREB binding sites on 2×*SOM-CRE-CAT* may lead to the reduction of the *CAT* gene expression we observed with TPA.

Fig. 2 shows that the co-transfection of expression vectors of c-*fos* and c-*jun* which form the most potent AP-1 complex has no effect on 8-Br-cAMP-stimulated gene expression. As gene expression mediated by AP-1 molecules from the CREs of 2×*SOM-CRE-CAT* may be responsible for the increased CAT expression we also transfected the *junB* gene. The JUNB protein is a quite poor transactivator [15] and often even blocks AP-1-mediated transcription [9]. Also this molecule did not block 8-Br-cAMP-mediated stimulation of gene transcription (Fig. 2), indicating that competition for DNA binding sites is not the mechanism that mediates the suppression of the PKA pathway by TPA. Control experiments with 2×*SOM-CRE-CAT* and AP-1 in the absence of 8-Br-cAMP showed that AP-1 alone could not activate *CAT* transcription from this reporter plasmid (data not shown).

We also tested the effect of TPA on the PKA catalytic subunit. Fig. 2 (lower panels) shows that in contrast to thapsigargin TPA cannot block the activity of the PKA catalytic subunit and that this molecule is not the target of TPA-induced suppression of the PKA pathway. TPA therefore must act upstream of PKA activation.

We further tested the influence of 8-Br-cAMP on TPA and thapsigargin-stimulated transcription using the plasmid

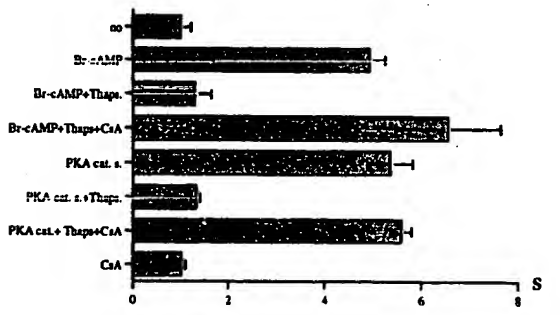

Fig. 1. Suppression of the activation (S) of gene expression by 8-Br-cAMP and the PKA catalytic subunit by the intracellular Ca^{2+} mobilizer thapsigargin in L4 fibroblast cells, using the reporter plasmid 2×*SOM-CRE-CAT*. The inducing and repressing agents are given on the left. CAT activity obtained from untreated cells (no) is set as 1.0. Standard deviation is indicated by error bars.

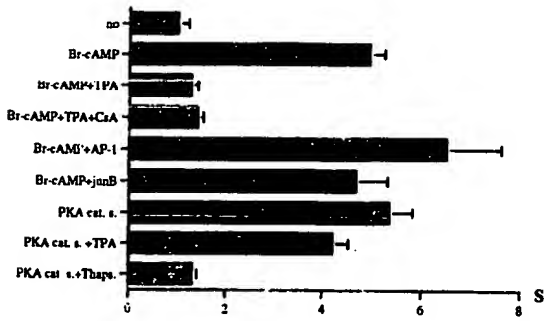

Fig. 2. The effect of TPA, AP-1, junB and CsA on 8-Br-cAMP and PKA catalytic subunit-stimulated gene transcription from the 2×*SOM-CRE-CAT* reporter plasmid. The stimulation factors (S) have been obtained as described in Fig. 1.

5×*TRE-CAT* which contains five AP-1 binding sites (TRE) in front of the *CAT* reporter gene. We found that 8-Br-cAMP cannot suppress the PKC- and Ca^{2+}-dependent signalling pathways (Fig. 3). This result implies that there is no PKA-regulated pathway which is able to block the action of the PKC and Ca^{2+} pathways in L4 cells.

4. Discussion

Our results show that there is in L4 fibroblasts an antagonism between the PKA signalling pathway on the one side and the PKC and Ca^{2+} signalling pathways on the other side. This antagonism is not reciprocal, as the latter two pathways dominate over the PKA pathway. The bias between these pathways is not due to the concentrations of the agents we used, as 1 mM 8-Br-cAMP turned out to be the optimal concentration, and concentrations higher than 1 mM 8-Br-cAMP reduced the cell growth and had a lower stimulating effect on gene transcription.

The dominance of the Ca^{2+} pathway over the PKA pathway is mediated by PP-2B as the PP-2B inhibitor CsA totally blocked the effect of the Ca^{2+} mobilizer thapsigargin. Our experiments also show that the stimulating effect of the PKA catalytic subunit can be blocked by intracellular Ca^{2+}, indicating that the catalytic subunit is one of the targets of the repression of the PKA pathway by high intracellular Ca^{2+} levels. PP-2B itself can dephosphorylate the regulatory subunit of PKA and thus favours the formation of the inactive PKA holoenzyme [16]. Eventually PP-2B does not only exert its effect by dephosphorylation of the catalytic subunit of PKA, but may also act in the same way that has been established for the glycogen metabolism [16,17] where PP-2B also de-represses the activity of the phosphatase PP-1 by inactivation of the PP-1 inhibitor 1 (I-1). PP-1 in turn dephosphorylates and inactivates substrates of PKA such as the transcription factor CREB [18]. A similar mechanism has also been found in dopaminoceptive neurons where the firing rate of the neurons is decreased by dopamine acting through cyclic AMP. This effect is overcome by glutamate acting via Ca^{2+} and this mechanism also involves derepression of PP-1. In this system DARPP 32, a homologue of I-1, is inactivated by PP2B [19,20] leading to de-repression of PP-1 which in turn inactivates substrates of PKA.

In their ability to repress PKA-dependent gene expression by Ca^{2+}, L4 fibroblast cells differ markedly from other cell types [21–23] which activate the PKA pathway by Ca^{2+}

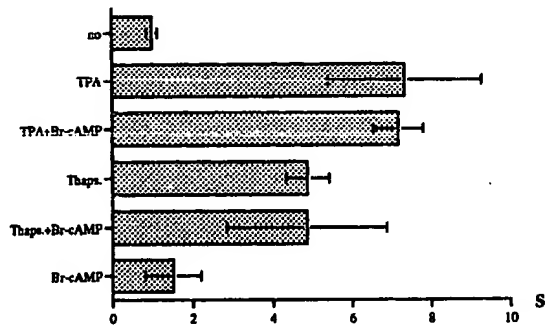

Fig. 3. The influence of 8-Br-cAMP on the TPA- and thapsigargin-induced gene transcription from the reporter plasmid 5×*TRE-CAT*. The stimulation factors (S) have been obtained as described in Fig. 1.

through the Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). We suppose that the PP-2B activity in L4 cells is much higher than the one of CaM kinase II. The different activity of certain protein kinases or phosphatases may explain the phenomenon that different signalling pathways synergize in some cell types, whereas they antagonize in others [1].

PKC activation suppresses the stimulating PKA effect in another way than Ca^{2+}. The fact that CsA does not relieve the suppressing effect of the PKC pathway on the PKA activation by 8-Br-cAMP excludes that PP-2B is involved in suppression of the PKA pathway by TPA. This conclusion is corroborated by the finding that the effect of the PKA catalytic subunit is not suppressed by the PKC pathway.

The suppression of the PKA pathway by PKC activators is also not effected on the level of transcription factors, as neither AP-1 (cJUN/cFOS heterodimer) nor JUNB can suppress the stimulation of gene transcription by the PKA activator 8-Br-cAMP. Therefore it seems that these and PKA-activated transcription factors do not compete for DNA binding sites or co-factors, as it has been shown in other systems [13,24]. This result also shows that AP-1 and JUNB do not activate another activity which blocks the PKA pathway.

In summary our results show that inhibition of the PKA pathway occurs upstream of the PKA catalytic subunit and downstream of adenylate cyclase. Our findings leave only the PKA holoenzyme as a target for the PKC-mediated repression. This assumption is corroborated by the results of Gallo et al. [25] who have shown that TPA blocks the dissociation of the PKA holoenzyme and the translocation of the catalytic subunit into the nucleus.

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Thank you

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Review

Coupling gene expression to cAMP signalling: role of CREB and CREM

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Abstract

Several endocrine and neuronal functions are governed by the cAMP-dependent pathway. Transcriptional regulation upon stimulation of this pathway is mediated by a family of cAMP-responsive nuclear factors. This family consists of a large number of members, which may act as activators or repressors. These factors contain the basic domain/leucine zipper motifs and bind as dimers to cAMP-response elements (CRE). CRE-binding protein (CREBs) function is modulated by phosphorylation by several kinases. Direct activation of gene expression by CREB requires phosphorylation by the cAMP-dependent PKA to serine 133. Among the repressors, ICER (Inducible cAMP Early Repressor) deserves special mention. ICER is generated from an alternative CREM promoter and is the only inducible CRE-binding protein. ICER negatively autoregulates the alternative promoter, generating a feedback loop. ICER expression is tissue specific and developmentally regulated. The kinetics of ICER expression are characteristic of an early response gene.

CREM plays a key physiological and developmental role within the hypothalamic–pituitary–gonadal axis. The transcriptional activator CREM is highly expressed in postmeiotic cells. The role of CREM in spermiogenesis was addressed using CREM knock-out mice. Spermatogenesis stops at the first step of spermiogenesis in the mutants and there is a significant increase in apoptotic germ cells. This phenotype is reminiscent of cases of human infertility.

ICER is regulated in a circadian manner in the pineal gland, the site of the hormone melatonin production. This night–day oscillation is driven by the endogenous clock (located in the suprachiasmatic nucleus). The synthesis of melatonin is regulated by a rate-limiting enzyme, serotonin *N*-acetyltransferase (NAT). Analysis of the CREM-null mice and of the promoter of the NAT gene revealed that ICER controls the amplitude and rhythmicity of NAT, and thus the oscillation in the hormonal synthesis of melatonin.

Keywords: Cyclic AMP; CREB; CREM; Phosphorylation; Autoregulation

1. Introduction

The regulation of gene expression by specific signal transduction pathways is tightly connected to the cell phenotype and, conversely, the response elicited by a given transduction pathway varies depending on the cell type. Several molecules implicated in intracellular signalling are encoded by oncogenes, directly linking their possible aberrant expression to cellular transformation or

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altered proliferation. A complete analysis of these processes will help to unravel the profound changes that cause cancer and, by the same token, understand the physiology of normal growth. A fundamental stride has been the discovery that many transcription factors constitute final targets of specific transduction pathways. Many distinct kinases have been shown to directly or indirectly modulate the activity of various nuclear factors (Karin and Hunter, 1995).

The activity of transcription factor AP-1 may be increased by inducing c-*fos* gene transcription, a process mediated by the ERK-1 and -2 mitogen-activated protein (MAP) kinases, which directly phosphorylate the transcription factor Elk-1/TCF, which then binds to the c-*fos* serum response element (Treisman, 1996). Alternatively, AP-1 activity maybe be enhanced by direct phosphorylation of Jun by a different type of MAPKs, the stress-activated protein kinases (JNK/SAPK) (Davis, 1994). Transcription factor ATF-2, a dimerization partner of Jun, is also a target of the JNK kinase (Hazzalin et al., 1996). Interestingly, ATF-2 was first cloned as a member of the ATF/CREB family of transcription factors and was shown to bind to cAMP-responsive elements (CREs) (Hai et al., 1989). The ATF/CREB family includes several members, of which only the CREB, CREM and ATF-1 gene products have been shown to be directly phosphoryalted by the cAMP-dependent protein kinase A (Sassone-Corsi, 1995). Cross-talk between the mitogenic signalling pathways and cAMP-responsive transcription has been established (Ginty et al., 1994), which reinforces the notion of converging signalling within the PKA and PKC pathways in the cytoplasm (Cambier et al., 1987; Yoshimasa et al., 1987; Frodin et al., 1994) and in the nucleus (Masquilier and Sassone-Corsi, 1992).

An important example of signalling cross-talk in the nucleus involves the pathway coupled to the NGF receptor, Trk, which results in the activation of several kinases. Trk is a receptor tyrosine kinase which, once activated, stimulates the activity of the small GTP-binding protein Ras (Gomez and Cohen, 1991). Activation of Ras triggers the MAPK pathway, which includes the MAP kinase kinase (MEK) and the ribosomal S6 kinase $pp90^{rsk}$ (Cobb and Goldsmith, 1995). Interestingly, constitutively activated expression of MAPK and MEK is sufficient to induce neurite outgrowth in PC12 cells (Cowley et al., 1994; Fukuda et al., 1995), indicating a direct role of this pathway in eliciting the changes in gene expression required for the neuronal differentiation program. Although MAPK and MEK have not been shown to directly phosphorylate CREB, the use of cells expressing a dominant-interfering Ras mutant has revealed the involvement of this pathway for CREB phosphorylation upon NGF-induction (Ginty et al., 1994). Indeed, the involvement of a CREB-kinase which could have characteristics similar to $pp90^{rsk}$ has been proposed (Fig. 1). $pp90^{rsk}$ is likely to be responsible for CREB phosphorylation in human melanocytes (Böhm et al., 1995), while the other member of the RSK family, $p70^{s6k}$, also possesses CREB phosphorylation activity (de Groot et al., 1994). Thus, two different signalling pathways may converge to modulate gene expression via the same transcriptional regulator, CREB (Fig. 1). Finally, CREB has been shown to be phosphorylated upon activation of the stress pathway involving the p38/MAPKAP2 kinases (Tan et al., 1996).

The complexity of the signalling pathways controlling transcription factors is a demonstration of the pleiotropic functions played by these molecules in the regulation of physiology and metabolism. Here we will focus primarily on the targets of the cAMP-mediated transduction response and their function within the neuroendocrine response.

2. Phosphorylation: a prerequisite for activation

Intracellular levels of cAMP are regulated primarily by adenylyl cyclase. This enzyme is, in turn, modulated by various extracellular stimuli mediated by receptors and their interaction with G proteins (McKnight et al., 1988). The binding of a specific ligand to a receptor results in the activation or inhibition of the cAMP-dependent pathway, ultimately affecting the transcriptional

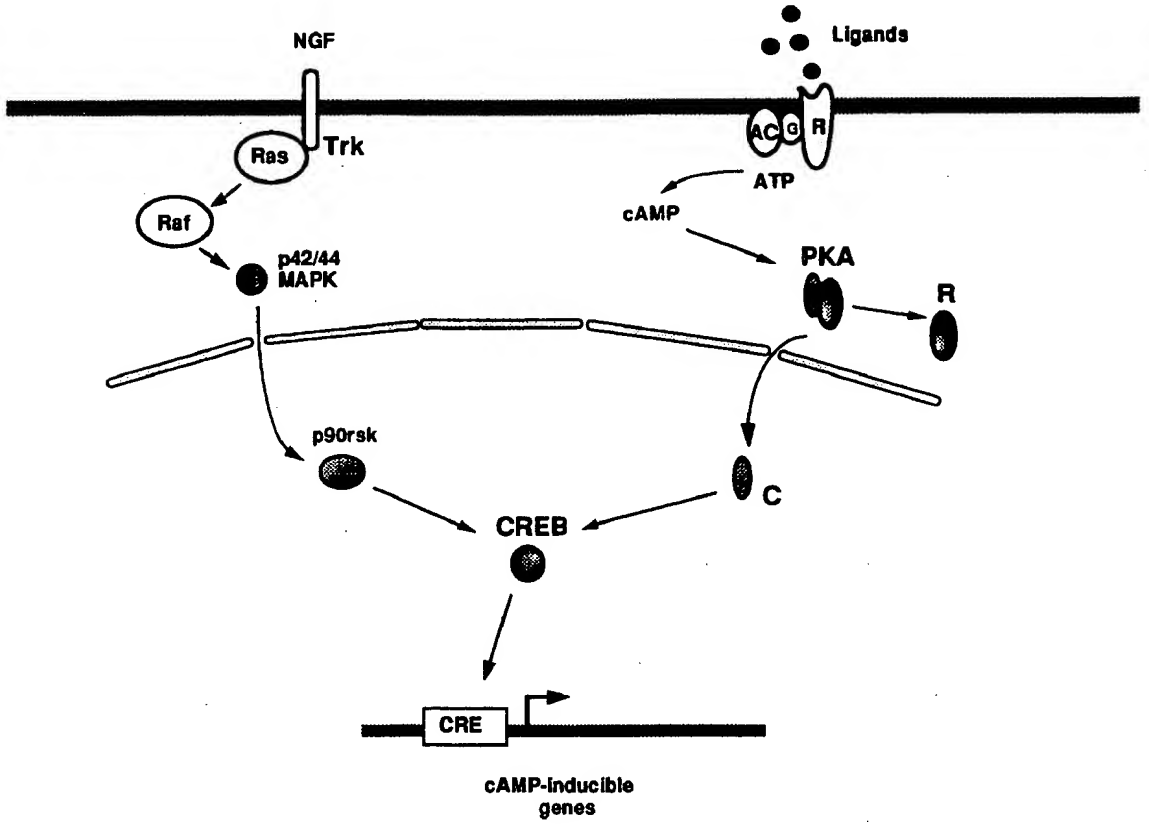

Fig. 1. Cross-talk in signal transduction. Schematic representation of the route whereby ligands at the cell surface interact with membrane receptors (R) and thereby result in altered gene expression upon activation of the cAMP signal transduction pathway. Ligand binding activates coupled G-proteins (G) which, in turn, stimulate the activity of the membrane-associated adenylyl cyclase (AC). This converts ATP to cAMP, which causes the dissociation of the inactive tetrameric protein kinase A (PKA) complex into the active catalytic subunits and the regulatory subunits. Catalytic subunits (C) migrate into the nucleus, where they phosphorylate and thereby activate transcriptional activators such as CREB. CREB then interacts as a dimer with the cAMP response enhancer element (CRE) found in the promoters of several cAMP-responsive genes to activate transcription. CREB phosphorylation may be obtained also by activation of the NGF (Nerve Growth Factor) tyrosine kinase receptor Trk. This pathway involves the Ras–Raf signalling cascade and results in the activation of the RSK class of kinases. CREB can be phosphorylated at the same PKA-phosphoacceptor site (Ser-133) by $p90^{rsk}$. This phosphorylation event may result in the activation of cAMP-responsive gene expression via a cAMP-independent signalling cascade.

regulation of various genes through distinct promoter-responsive sites. Increased cAMP levels directly affect the function of the tetrameric protein kinase A (PKA) complex. Binding of cAMP to two PKA regulatory subunits releases the catalytic subunits, enabling them to phosphorylate target proteins. These are translocated from cytoplasmic and Golgi complex anchoring sites and phosphorylate a number of cytoplasmic and nuclear proteins on serines in the context X-Arg-Arg-X-Ser-X (McKnight et al., 1988; Roesler et al., 1988). A number of isoforms for both the regulatory and catalytic subunits have been identified, suggesting a further level of complexity in this response (McKnight et al., 1988). In the nucleus, the phosphorylation state of transcription factors and related proteins appears to directly modulate their function and thus the expression of cAMP-inducible genes. Thus, there is a direct link between the activation of G-coupled membrane receptors and CRE-mediated gene expression.

The analysis of regulatory sequences of several genes allowed the identification of promoter elements which mediate the transcriptional response to increased levels of intracellular cAMP (Lalli and Sassone-Corsi, 1994). A number of sequences have been identified, of which the best characterised is the CRE. A consensus CRE site constitutes an 8 bp palindromic sequence (TGACGTCA) (Sassone-Corsi, 1988; Ziff, 1990). Several genes which are regulated by a variety of endocrinological stimuli contain similar sequences in their promoter regions, although at different positions. A comparison of the CRE sequences identified to date, shows that the 5′-half of the palindrome, TGACG is the best conserved, differently from the 3′ TCA motif (Sassone-Corsi, 1995).

The first CRE-binding factor to be characterised was CREB (CRE-binding protein; Hoeffler et al., 1988) but subsequently several additional CRE-binding factors have been identified and the corresponding gene cloned. Most of the CRE-binding proteins were identified by screening a variety of cDNA expression libraries with CRE and ATF sites (Hai et al., 1989; Foulkes et al., 1991). All these proteins belong to the bZip transcription factor class, while outside of the bZip region, sequence homology between these factors is relatively poor. Various different factors of the CREB/ATF family are able to heterodimerize with each other but only in certain combinations. A "dimerization code" exists, which seems to be a property of the leucine zipper structure of each factor.

CRE-binding proteins may act as both activators and repressors of transcription. The activators mediate transcriptional induction upon their phosphorylation by PKA (Gonzalez and Montminy, 1989; Rehfuss et al., 1991; de Groot et al., 1993; Sassone-Corsi, 1995). Their expression is constitutive and widely distributed in various tissues in a housekeeping fashion. Among the repressors, the cAMP-inducible ICER (Inducible cAMP Early Repressor) product deserves special mention. It is generated from a cAMP-inducible alternative promoter of the CREM gene (Molina et al., 1993; Stehle et al., 1993). Thus, ICER is an early response CRE-binding factor and is involved in the dynamics of cAMP-responsive transcription (Lamas et al., 1996).

3. Interaction with CBP

Further steps towards an understanding of the mechanism of action of the P-box have arisen with the identification of a 265 K, 2441 amino acid protein, CBP (CREB-binding protein) that is able to interact specifically with the phosphorylated CREB P-box domain (Chrivia et al., 1993). The CBP sequence reveals two zinc finger domains, a glutamine-rich domain at its C-terminus and a single consensus PKA recognition site. Phosphorylation of Ser-133 promotes binding to CBP and consequently the interaction with TFIIB, a general transcription factor involved in RNA polymerase II activity (Kwok et al., 1994). Thus, CBP may act as a link between CREB and the transcription pre-initiation complex. This interaction may reqire some RNA polymerase II cofactors, such as TAF110. Finally, the adenoviral E1A oncoprotein-associated p300, which is thought to play a role in preventing the cell cycle G0/G1 transition, is structurally very closely related to CBP (Arany et al., 1995). Both CBP and p300 appear to have intrinsic activating properties which are inhibited by the E1A protein (Arany et al., 1995). Thus, it is clear that studies of the transcriptional activation domain of CRE-binding bZip factors continue to provide important insights into the function of transcription factors in general.

4. Mechanisms of repression

Dephosphorylation appears to represent a key mechanism in the negative regulation of CREB activation function. It has been proposed that a mechanism to explain the attenuation of CREB activity following induction by forskolin is dephosphorylation by specific phosphatases (Hagiwara et al., 1992). After the initial burst of phosphorylation in response to cAMP, CREB is dephosphorylated *in vivo* by protein phosphatase-

1 (PP-1). However, the situation is more complex, since it has been shown that both PP-1 and PP-2A can dephosphorylate CREB *in vitro* (Nichols et al., 1992) resulting in an apparent decreased binding to low-affinity CRE sites *in vitro*. Therefore, the precise role of PP-1 and PP-2A in the dephosphorylation of CREB remains to be determined.

The discovery of the CREM gene opened a new dimension in the study of the transcriptional response to cAMP (Foulkes and Sassone-Corsi, 1992). The dynamic and versatile pattern of CREM expression combined with its tissue- and developmental-specific pattern, contrasts with that of the remaining members of the CRE-binding factor family, which seem to be constant and ubiquitous (Hai et al., 1989; Borrelli et al., 1992). These features offered the first clue that CREM occupied a priviliged position amongst this group of factors.

Various studies have established that differential transcript processing is central to the regulation of CREM expression. The importance of this mechanism is reinforced by the fact that all the CREM isoforms which incorporate the P-box exons (Fig. 2) are generated from a GC-rich promoter (P1), which has been shown to behave as a housekeeping promoter directing a non-inducible pattern of expression (Molina et al., 1993; Stehle et al., 1993).

5. An inducible repressor: ICER

An alternative promoter lying within an intron near the 3′ end of the CREM gene, directs the transcription of a truncated product, termed ICER (Inducible cAMP Early Repressor) (Molina et al., 1993; Stehle et al., 1993). The ICER open reading frame is constituted by the C-terminal segment of CREM (Fig. 2). The predicted open reading frame encodes a small protein of 120 amino acids with an expected molecular weight of 13.4 kDa. This protein, compared with the previously described CREM isoforms, essentially consists of only the DNA-binding domain, which consists of the leucine zipper and basic region. The unique structure of ICER is suggestive of its function and makes it one of the smallest transcription factors ever described (Molina et al., 1993; Stehle et al., 1993).

The intact DNA-binding domain directs specific ICER binding to a consensus CRE el-

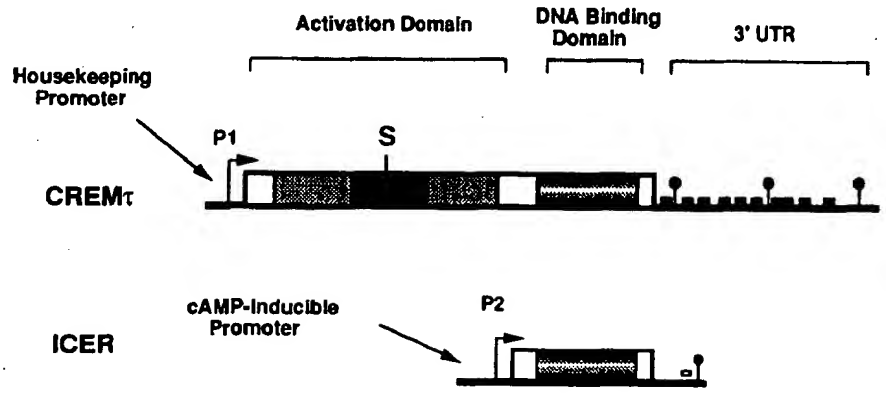

Fig. 2. Activators and repressors from the same gene. Schematic representation of the CREM gene. The various activator and repressor CREM isoforms are indicated. The P1 promoter is GC-rich and directs a non-inducible pattern of expression of the activator CREMτ, which has a structure similar to CREB. The P2 promoter is strongly inducible by activation of the cAMP-dependent signalling pathway and directs the synthesis of the powerful repressor ICER. In germ cells the abundant CREMτ transcript is polyadenylated at an alternative site which confers increased stability. Schematic representation of the 3′ untranslated region (3′ UTR). The three polyadenylation signals (polyA) are non-canonical and are indicated by a stem-loop; each AUUUA destabiliser is represented by a small square. Use of the testis-specific site generates a transcript with a truncated 3′ untranslated region and only one instability element. This transcript is intrinsically more stable.

ement. Importantly, ICER is able to heterodimerize with the other CREM proteins and with CREB. ICER functions as a powerful repressor of cAMP-induced transcription in transfection assays using an extensive range of reporter plasmids carrying individual CRE elements or cAMP-inducible promoter fragments (Molina et al., 1993). Interestingly, ICER-mediated repression is obtained at substoichiometric concentrations, similarly to the previously described CREM antagonists (Laoide et al., 1993). ICER escapes from PKA-dependent phosphorylation and thus constitutes a new category of CRE binding factor, for which the principle determinant of their activity is their intracellular concentration and not their degree of phosphorylation.

The expression of ICER was first described in the pineal gland, where it is the subject of a dramatic circadian pattern of expression (Stehle et al., 1993). Additional data implicate dynamic ICER expression as a general feature of neuroendocrine systems (Lamas and Sassone-Corsi, 1996). An important feature about ICER is its inducibility. This makes ICER the only CRE-binding protein whose function is physiologically regulated by altering its cellular concentration.

6. CREM is an early response gene

During studies of CREM expression within the neuroendocrine system, an unexpected new facet emerged: namely the transcription of the CREM gene is inducible by cAMP (Molina et al., 1993). Furthermore, the kinetics of this induction is that of an early response gene (Verma and Sassone-Corsi, 1987). This important finding further reinforces the notion that CREM products play a fulcral role in the nuclear response to cAMP, since the expression of no other CRE-binding factor has been shown to be inducible to date. For example, the recently characterised CREB promoter is GC-rich and reminiscent of the promoters of constitutively expressed, housekeeping genes (Meyer et al., 1993). Similarly, the promoter which directs expression of the other CREM isoforms (P1) is not cAMP inducible (Molina et al., 1993).

Clues that the CREM gene was cAMP inducible first came from the demonstration that adrenergic signals direct CREM transcription in the pineal gland (Stehle et al., 1993). The inducibility phenomenon was then characterised in detail in the pituitary corticotroph cell line AtT20. In unstimulated cells the level of CREM transcript is below the threshold of detectability. However, upon treatment with forskolin (or other cAMP analogs), within 30 min there is a rapid increase in CREM transcript levels, which peak after 2 h and then progressively decline to basal levels by 5 h. This characteristic expression profile classifies CREM as an "early response gene" and thus directly implicates the cAMP pathway in the cell's early response for the first time. CREM inducibility is specific for the cAMP pathway, since it is not inducible by TPA or dexamethasone treatment (Molina et al., 1993).

The 5′ end of the ICER clones correspond to an alternative transcription start site. The start of transcription, which identifies the so-called P2 promoter, is within the 10 kb intron which is C-terminal to the Q2 glutamine-rich domain exon. In contrast to the promoter which generates all the previously characterised CREM isoforms (P1) which is GC-rich and not inducible by cAMP (unpublished results), the P2 promoter has a normal A-T and G-C content and is strongly inducible by cAMP. It contains two pairs of closely spaced CRE elements organized in tandem, where the separation between each pair is only three nucleotides. These features make P2 unique amongst cAMP-regulated promoters and are suggestive of cooperative interactions among the factors binding to these sites.

7. A negative autoregulatory loop

Upon cotreatment with cycloheximide, the kinetics of CREM gene induction by forskolin are altered in that there is a significant delay in the post-induction decrease in the transcript; elevated levels persist for as long as 12 h. This implicates a *de novo* synthesised factor which might downregulate CREM transcription (Molina et al., 1993). This observation, combined with the presence of

CRE elements in the P2 promoter, suggested that the transient nature of the inducibility could be due to ICER. Consistently, the CRE elements in the P2 promoter have been shown to bind to the ICER proteins. Detailed studies have demonstrated that the ICER promoter is indeed a target for ICER negative regulation (Molina et al., 1993; Lamas et al., 1996). Thus, there exists a negative autoregulatory mechanism controlling ICER expression. The CREM feedback loop predicts the presence of a refractory inducibility period in the gene's transcription (see Fig. 3; Sassone-Corsi, 1994).

8. Role of CREM in spermatogenesis

CREM is a highly abundant transcript in adult testis, while in prepubertal animals it is expressed at very low levels. Thus, CREM is the subject of a developmental switch in expression in testis (Foulkes et al., 1992). Further charaterisation revealed that the abundant CREM transcript encodes the activator exclusively, while in prepubertal testis only the repressor forms were detected at low levels. Thus, the CREM developmental switch also constitutes a reversal of function (Foulkes and Sassone-Corsi, 1992).

Spermatogenesis is a process occurring in a precise and coordinated manner within the seminiferous tubules (Jégou, 1993). During this entire developmental process the germ cells are maintained in intimate contact with the somatic Sertoli cells. As the spermatogonia mature, they move from the periphery towards the lumen of the tubule until the mature spermatozoa are conducted from the lumen to the collecting ducts.

CREM activator protein is detected in mature germ cells, such as round spermatids, which have undergone meiosis (Delmas et al., 1993). Thus, CREM transactivator function must be restricted to the late phase of transcription before the com-

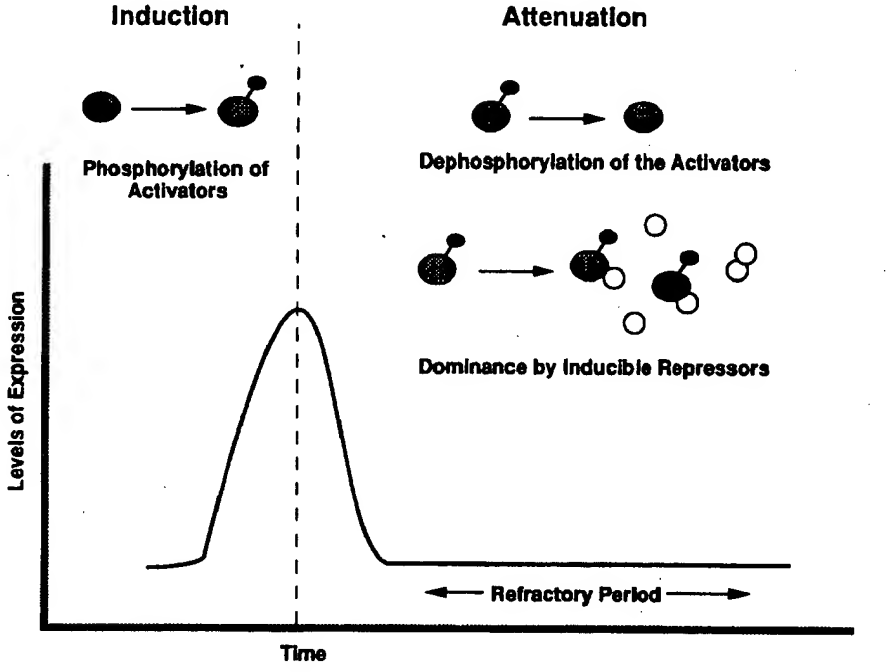

Fig. 3. Kinetics of CREM inducibility. After the induction phase, owing to the phosphorylation of the activators (i.e. CREB), expression is attenuated by at least two mechanisms: (a) dephosphorylation of the activators by some specific phosphatases; (b) negative autoregulation by the *de novo* synthesised ICER repressor on the P2 promoter (see Fig. 2) (Molina et al., 1993; Sassone-Corsi, 1994).

paction of the DNA. Interestingly, several genes have been identified which are transcribed at the time of appearance of the CREM protein and which include CRE-like sequences in their promoter regions. Several lines of evidence demonstrate that CREM constitutes the first step of a transcriptional cascade which is responsible for the activation of several germ-specific genes. To date, at least three genes, RT7 (Delmas et al., 1993), transition protein-1 (Kistler et al., 1994) and claspermin (Sun et al., 1995) have been shown to be targets of CREM-mediated transactivation in germ cells. Importantly, the dramatic increase in the levels of CREM protein correlates with its concomitant phosphorylation at serine 117 by a cAMP-stimulated PKA activity in round spermatid extracts (Delmas et al., 1993). Thus, CREM appears to participate in the testis-specific promoter activation of numerous haploid-expressed genes (Sassone-Corsi, 1997).

A remarkable aspect of the CREM developmental switch in germ cells is constituted by its exquisite hormonal regulation. The spermatogenic differentiation program is under the tight control of the hypothalamic–pituitary axis (Jégou, 1993). The regulation of CREM function in testis seems to be intricately linked to FSH, both at the level of the control of transcript processing and at the level of protein activity. For example, surgical removal of the pituitary gland leads to the loss of CREM expression in the rat adult testis (Foulkes et al., 1993). Furthermore hypophysectomisation in prepubertal animals, prevents the switch in CREM expression at the pachytene spermatocyte stage, thus implicating the pituitary directly in the maintenance of, as well as the switch to high levels of CREM expression. Injections of FSH lead to a rapid and significant induction of the CREM transcript. The hormonal induction of CREM by FSH is not transcriptional, as expected by the housekeeping nature of the P1 promoter. Instead, by a mechanism of alternative polyadenylation, AUUUA destabiliser elements present in the 3′ untranslated region of the gene are excluded, dramatically increasing the stability of the CREM message (Fig. 2). CREM is the first example of a gene whose expression is modulated by a pituitary hormone during spermatogenesis (Foulkes et al., 1993). The implication of these findings is that hormones can regulate gene expression at the level of RNA processing and stability.

To address the role of CREM in development and in physiological processes we generated mutant mice with a gene disrupted by homologous recombination in mouse embryonic stem cells (Nantel et al., 1996). We constructed a targeting vector containing a CREM genomic fragment in which a portion of the 3′-terminal exon encoding the DNA-binding domain was deleted and replaced by a PGK-neomycin cassette. The selection of the construct was dictated by the need to inactivate all the numerous CREM and ICER isoforms (Laoide et al., 1993; Stehle et al., 1993). Reduced fertility was observed in the breeding of the heterozygous mice. Comparison of the homozygous CREM-deficient mice with their normal littermates showed no macroscopic physical aberrations or reduction in body weight. Analysis of internal organs revealed no apparent changes in their structure as compared with wild-type mice. However, the testes of the CREM-deficient mice displayed a reduction of 20–25% in their weight. Analysis of the seminal fluid of heterozygous mice compared with normal littermates demonstrated a 46% reduction in the overall number of spermatozoa, a 35% decrease in the ratio of motile spermatozoa, and a 2-fold increase in the number of spermatozoa with aberrant structures. Most of the aberrant spermatozoa were characterized by a kink and bubble-like structure midway along the tail. Strikingly, analysis of the seminal fluid from homozygous CREM-deficient mice revealed a complete absence of spermatozoa. This result demonstrates a dramatic impairement of spermatogenesis in the CREM-deficient mice. The homozygous female mice were fertile and displayed apparently normal ovary structure.

To determine the nature of the sperm deficiency in the CREM-deficient mice, we performed a detailed anatomical analysis of the seminiferous epithelium. Consecutive spermatogenic cycles are classically depicted as waves of differentiating germ cells within each tubule

(Parvinen, 1993). In the mouse, each wave is divided into 12 stages, each representing a specific cellular association. Tubular segments containing postmeiotic germ cells which undergo spermiogenesis appear as dark sections under transillumination because of the higher DNA compaction of these haploid cells (Parvinen, 1993). Tubuli from CREM-deficient mice display a 20–30% reduced diameter and completely lack the normal spermatogenic wave and the corresponding dark sections. Squash preparations from consecutive segments of the seminiferous epithelium demonstrate that spermatogenesis in the CREM-deficient mice is interrupted at the stage of very early spermatids. Neither elongating spermatids, nor spermatozoa, are observed, while somatic Sertoli cells appear to be normal.

9. Role of CREM in circadian rhythms

Crucial elements for the synchronization of biological rhythms in mammals are the pineal gland (Tamarkin et al., 1985) and the suprachiasmatic nucleus (SCN) (Moore, 1983). Environmental lighting conditions are transduced by the pineal gland from a neuronal to an endocrine message, the rhythmic secretion of melatonin (Tamarkin et al., 1985). This hormone synthesis is controlled by the SCN, being elevated at night and low during the day (Moore, 1983). The cAMP-dependent signal transduction pathway serves as a relay to stimulate melatonin synthesis. Thus, from neuronal pathways which include the retina and the SCN, the pineal gland acts as a temporal regulator of the hypothalamic–pituitary–gonadal axis (Tamarkin et al., 1985).

The study of CREM expression in the rat brain indicated a specific pattern of expression (Mellström et al., 1993). Analysis of CREM expression in the pineal gland has revealed a dramatic day–night regulation, with peak during the night. The CREM isoform in the pineal gland corresponds to ICER, the early response repressor known to be cAMP-inducible in endocrine cells (Stehle et al., 1993). The transcript shows a very characteristic and reproducible kinetic of expression. It appears likely that the autoregulatory loop shown to control ICER transient inducibility would also play a role in the day–night cyclic expression in the pineal gland.

The mechanism controlling this pattern of ICER expression was determined and found to require clock-distal elements. Indeed, it is known that at night, postganglionic fibers originating from the superior cervical ganglia (SCG) release norepinephrine, which in turn regulates melatonin synthesis via adrenergic receptors. These analyses have shown that signals from the SCN direct the induction of CREM expression (Stehle et al., 1993).

The question of possible targets for downregulation by ICER in the gland is of particular interest. It has been proposed that a reasonable target could be the enzyme which catalyses the rate-limiting step of melatonin synthesis, namely *N*-acetyl transferase (NAT) or factors which regulate its activity. Recent results indicate that this is indeed the case. The NAT promoter was shown to contain a CRE which binds ICER with high affinity. In addition, the amplitude of NAT oscillation in CREM-deficient mice was shown to be altered with respect to wild-type animals, demonstrating that NAT is a direct target of CREM (Foulkes et al., 1996).

Another important finding concerning the role of CRE-binding factors in circadian rhythms concerns the cyclic phosphorylation of CREB in the suprachiasmatic nucleus (Ginty et al., 1993). During the night, upon light stimuli which phase-shift the clock, CREB appears to be efficiently phosphorylated by an SCN-endogenous kinase at the serine 133 residue. Phosphorylation at this site turns CREB into an activator and may be obtained by a number of kinases (Lalli and Sassone-Corsi, 1994). While the nature of the SCN-endogenous kinase has not been established, it seems likely that it could be PKA (Ginty et al., 1993). This result would suggest a key role for this kinase or of a counteracting phosphatase in the regulation of the clock function. The target genes for the activated CREB in the SCN have yet to be established.

10. Conclusions and perspectives

The cAMP signal transduction pathway plays a key role in many biological processes. In the mammalian neuroendocrine system, it is central to the coordination of hormonal function. cAMP directs changes in gene expression and thereby effects long-term modulation. A great diversity of cAMP responsive transcription factors, notably CREM, seems to be a hallmark of this system. Many issues still remain to be explored in delineating the means by which CRE-binding proteins regulate complex phenomena such as memory formation and establishment. However, the abundance of molecular tools now available should aid this task. By understanding the precise mode of action of cAMP we should gain a more general insight into the molecular architecture which underlies physiology.

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Thank you

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The cAMP-Dependent Protein Kinase Regulates Transcription of the Dopamine β-Hydroxylase Gene

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Dopamine β-hydroxylase (DBH) catalyzes the conversion of dopamine to norepinephrine, and is expressed specifically in neurons and neuroendocrine cells that release norepinephrine and epinephrine. In the present study, we used DBH-expressing human neuroblastoma SK-N-BE(2)C and rat pheochromocytoma (PC12) cell lines to investigate the role of cAMP-dependent protein kinase (PKA) in transcriptional regulation of the DBH gene. Coexpression of the catalytic subunit of PKA (PKA_c) robustly stimulated the transcriptional activity of the DBH gene in a dose-dependent manner. Conversely, coexpression of a specific inhibitor of PKA abrogated forskolin- and cAMP-mediated but not phorbol ester–mediated transcriptional induction of DBH. Deletion of the cAMP response element (CRE) dramatically reduced the stimulatory effect of PKA, indicating that the CRE mediates the induction of DBH by PKA. In DBH-nonexpressing HeLa and C6 glioma cell lines, coexpression of PKA_c changed the transcriptional activity of the DBH promoter to a minimal degree, indicating that basal and PKA-mediated transcription of the DBH gene occur in a cell type–specific manner. Finally, both basal and cAMP-stimulated transcription of the DBH gene are diminished in three PKA-deficient PC12 cell lines, compared to wild-type cells. Based on these data, we conclude that PKA, via the CRE, plays an important role in basal and cAMP-inducible transcription, but is not required for phorbol ester–mediated induction, of the DBH gene in noradrenergic cells. The present results, together with previous evidence supporting a critical role for PKA in the transcriptional regulation of the tyrosine hydroxylase (TH) gene, suggest that the PKA pathway can regulate transcription of the TH and DBH genes in a coordinated fashion.

[Key words: cAMP, cAMP-dependent protein kinase (PKA), dopamine β-hydroxylase, transcriptional regulation, tyrosine hydroxylase, norepinephrine biosynthesis, cAMP response element, polypeptide inhibitor of PKA (PKI), primer extension, cotransfection analysis]

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The catecholamines, that is, dopamine, norepinephrine, and epinephrine, are differentially synthesized in subsets of catecholamine neurons and neuroendocrine cells in a cell-specific manner. Underlying this specificity of synthesis is cell-specific expression of the enzymes catalyzing catecholamine biosynthesis. For example, dopamine β-hydroxylase (DBH; EC 1.14.17.1) converts dopamine to norepinephrine (Kirshner and Goodall, 1957; Friedman and Kaufman, 1965). Its expression thus confers a noradrenergic or adrenergic phenotype on catecholamine-synthesizing cells. At present, the molecular mechanisms underlying tissue-specific expression of catecholamines and their biosynthesizing enzymes are not well understood.

Norepinephrine- and epinephrine-secreting cells regulate the level of tyrosine hydroxylase (TH) and DBH gene expression in response to a variety of transsynaptic, hormonal, and growth factor signals, including cAMP analogs (Sabban et al., 1983; Lewis et al., 1987; Lamouroux et al., 1993), nerve growth factor (Acheson et al., 1984; Badoyannis et al., 1991), glucocorticoids (Otten and Thoenen, 1976; K. T. Kim et al., 1993), reserpine (Faucon Biguet et al., 1986; Wessel and Joh, 1993), and immobilization stress (McMahon et al., 1992). TH and DBH expression change in the same direction in response to such stimuli, suggesting that common regulatory mechanisms influence transcription of the genes encoding these enzymes. Several lines of evidence from this laboratory support the hypothesis that TH and DBH share common molecular mechanisms of transcriptional control. For example, the cAMP response element (CRE) plays an important dual role in both basal and cAMP-stimulated transcription of the TH gene (K. S. Kim et al., 1993a). Similarly, the CRE is an essential positive genetic element for cell-specific and forskolin-stimulated expression of the human DBH gene (Ishiguro et al., 1993).

Recent data from this laboratory demonstrated that the PKA-signaling pathway regulates both the basal and cAMP-inducible transcription of the TH gene (K. S. Kim et al., 1993b, 1994). These observations prompted us to hypothesize that PKA also controls basal and cAMP-inducible transcription of the DBH gene, resulting in a coordinated regulation of the TH and DBH genes by PKA. The present study examined this possibility by transient transfection assays using reporter constructs containing 5′ flanking sequences of the human DBH gene fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. We tested the effects of coexpression of the catalytic subunit of PKA, or a specific peptide inhibitor of PKA, on the transcriptional activity of these reporter constructs in the human neuroblastoma SK-N-BE(2)C (DBH-expressing) and HeLa (DBH-nonex-

pressing) cell lines. In addition, we analyzed basal and cAMP-stimulated induction of DBH gene transcription in several PKA-deficient mutant PC12 lines. This study strongly suggests that PKA, acting via the CRE, plays a key role in the transcriptional regulation of the DBH gene in noradrenergic cells. These findings support the hypothesis that the PKA pathway functions as a common regulatory mechanism for DBH and TH gene transcription.

Materials and Methods

Cell culture and treatment with effector molecules. Human neuroblastoma cell lines SK-N-BE(2)C and SK-N-BE(2)M17, which express both tyrosine hydroxylase and dopamine β-hydroxylase (Ciccarone et al., 1989; Carroll et al., 1991; Ishiguro et al., 1993), and HeLa and C6 glioma cells, which do not express either enzyme, were maintained as described before (Ishiguro et al., 1993; K. S. Kim et al., 1993a). After 30–40 passages, cell cultures were restarted from early passage cells stored in liquid nitrogen. Stock solutions (1000×) of forskolin and the phorbol ester tetradecanoyl phorbol acetate (TPA) were prepared in dimethyl sulfoxide and added directly to the culture medium 16–18 hr before cells were harvested, to achieve final concentrations of 10 μM and 0.1 μg/ml, respectively. To inhibit phosphodiesterase activity, forskolin treatment was always paired with 3-isobutyryl-1-methylxanthine (IMX) to a final concentration of 0.5 mM. The intracellular activity of PKA was determined according to the published procedure (Roskoski, 1983), using the assay kit (GIBCO–Bethesda Research Labs). Mutant PC12 cell lines were rendered PKA deficient either by genetic manipulation (Ginty et al., 1991a; AB.11 and 123.7 cell lines) or chemical mutagenesis (Buskirk et al., 1985; A126-1B2 cell line). These mutant and wild-type PC12 cells were maintained and grown as described (K. S. Kim et al., 1993b).

Plasmids. The DBH-CAT reporter constructs 2.6CAT, 978CAT, and ΔCRE 978CAT were described previously (Fig. 1; Ishiguro et al., 1993). 2.6CAT contains the intact 2.6 kb upstream sequence of the human DBH gene fused to the chloramphenicol acetyl transferase gene. ΔCRE 978CAT contains 978 bp of the upstream sequence, in which 14 bases (−189 to −176), encompassing the CRE, were deleted. The heat-stable specific inhibitor of PKA (PKI) and its mutant form (PKI^m) were expressed using plasmids RSV-PKI and RSV-PKI^m (Day et al., 1989), generously provided by Dr. R. Maureer (University of Iowa). These plasmids utilize the promoter/enhancer region of the Rous sarcoma virus to direct expression of PKI. The expression plasmid for the catalytic subunit of PKA, PKA_c, was described before (Lee et al., 1990).

Transient transfection experiments. Transient transfection of the DBH-CAT reporter construct and effector molecules of PKA were performed by the calcium phosphate coprecipitation method as described before (Ishiguro et al., 1993; K. S. Kim et al., 1993a). When SK-N-BE(2)C or SK-N-BE(2)M17 cells reached approximately 50% confluence, each 60 mm dish was transfected with 2 μg of the reporter construct, varying amounts of the effector expression plasmid, 1 μg of pRSV-βgal, and PUC19 plasmid to a total DNA concentration of 5 μg. For HeLa and C6 glioma cells, twice as much DNA was used in transfection. To correct for differences in transfection efficiency among different DNA precipitates, CAT activity was normalized to the activity of β-galactosidase. The experiments described in Figure 3 utilized 2 μg of SV40-βgal for normalization. Assays of chloramphenicol acetyltransferase and β-galactosidase were performed as described (Ishiguro et al., 1993; K. S. Kim et al., 1993a).

Northern blot hybridization. To analyze the steady-state levels of mRNA of wild-type and mutant PC12 cells, polyA⁺ RNA was isolated by oligo(dT)-cellulose affinity column chromatography (Badley et al., 1988). A cDNA probe for the rat DBH gene, isolated in this laboratory (K. T. Kim et al., 1993), was used to identify the DBH message. Northern hybridization experiments were performed using 2 μg of polyA⁺ RNA per lane as described (K. S. Kim et al., 1993b). Levels of mRNA were quantitated and compared using a PhosphorImager and IMAGEQUANT software (Molecular Dynamics) and were normalized to those of a control gene, α-tubulin.

Primer extension analysis of fusion gene transcripts. To analyze the RNA products of the transient expression assays, a total of 15 μg of DNA containing 9 μg of the 2.6CAT plasmid and varying amounts of PKA_c was coprecipitated and transfected into cells grown to approximately 50% confluence in a 100 mm dish. The transcription initiation site of the reporter gene was determined by primer extension analysis as described (McKnight and Kingsbury, 1982), with the following modifications: polyA⁺ mRNA was isolated from SK-N-BE(2)C cells 36 hr after transfection as described above for the Northern hybridization experiments. An oligonucleotide (5′-CGGTGGTATATCCAGTG-3′) complementary to nucleotides 15–31 of the coding sequence of the CAT gene was end labeled by T4 polynucleotide kinase (NEB) using γ-^{32}P-ATP (6000 Ci mmol^{-1}; Amersham). Approximately 0.05 ng of labeled primer (specific activity, 2 × 10⁹ cpm/μg) was mixed with 4 μg of polyA⁺ RNA. Annealed primer was extended by 100 units of MMLV-reverse transcriptase (Bethesda Research Labs–GIBCO) at 37°C for 2 hr, in the presence of RNase inhibitor (U.S. Biochemical) and four deoxynucleotides. After RNase treatment, phenol/chloroform extraction, and EtOH precipitation, the final reaction mix was resuspended in 10 μl of loading gel buffer. Four microliters of each extension product were analyzed on an 8% polyacrylamide sequencing gel. A parallel dideoxy sequencing reaction was performed, utilizing the same oligonucleotide primer described above. An m13mp19 subclone containing the 1.3 kb SphI-EcoRI fragment from 978CAT plasmid (Ishiguro et al., 1993) served as the template. The gel was fixed, vacuum dried, exposed at −70°C for 36 hr with an intensifying screen, and visualized by autoradiography.

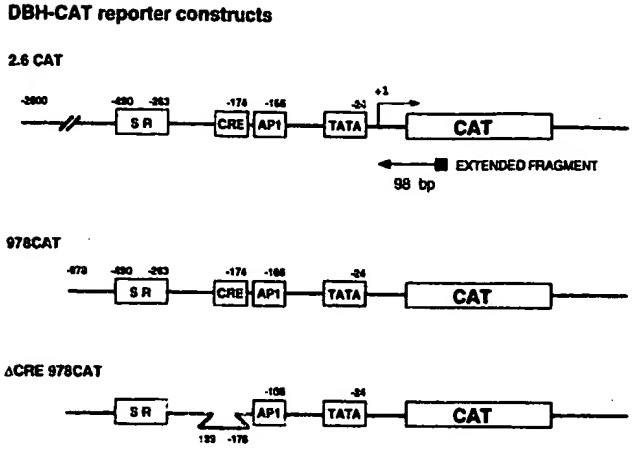

Figure 1. A schematic diagram of the DBH-CAT reporter constructs. *2.6 CAT* and *978CAT* contain 2.6 kb and 978 bp of the 5′ flanking sequence of the human DBH gene, respectively. Both sequences are capable of cell-specific transcription in cell culture systems (Ishiguro et al., 1993). *ΔCRE 978CAT* is identical to 978CAT, except 14 bp (−189 to −176), encompassing the CRE, has been deleted. Sequence motifs of the human DBH gene, previously identified and/or characterized (Kobayashi et al., 1989; Ishiguro et al., 1993; Lamouroux et al., 1993) and their relative position are represented by *numbered white boxes*. *SR* denotes the silencer region necessary for cell specificity of the DBH gene (Ishiguro et al., 1993). *+1* and the *bent arrow* denote the transcription initiation (CAP) site. The *black box* represents the oligonucleotide used in the primer extension and sequencing reactions.

Results

Expression of the catalytic subunit of PKA stimulates the transcriptional activity of the human DBH gene promoter in a cell type–specific manner

To define the role of PKA in transcriptional regulation of the DBH gene, three DBH-CAT fusion constructs were employed in this study as reporter plasmids. The 2.6CAT and 978CAT plasmids contain the intact 2600 bp and 978 bp upstream sequences of the human DBH gene, respectively. The ΔCRE 978CAT plasmid contains 978 bp of upstream sequence, with the deletion of 14 bp (−189 to −176) encompassing the CRE (Fig. 1). To analyze the role of PKA in transcriptional regulation of the DBH gene, an expression plasmid encoding PKA_c was cotransfected with the DBH-CAT fusion constructs into human

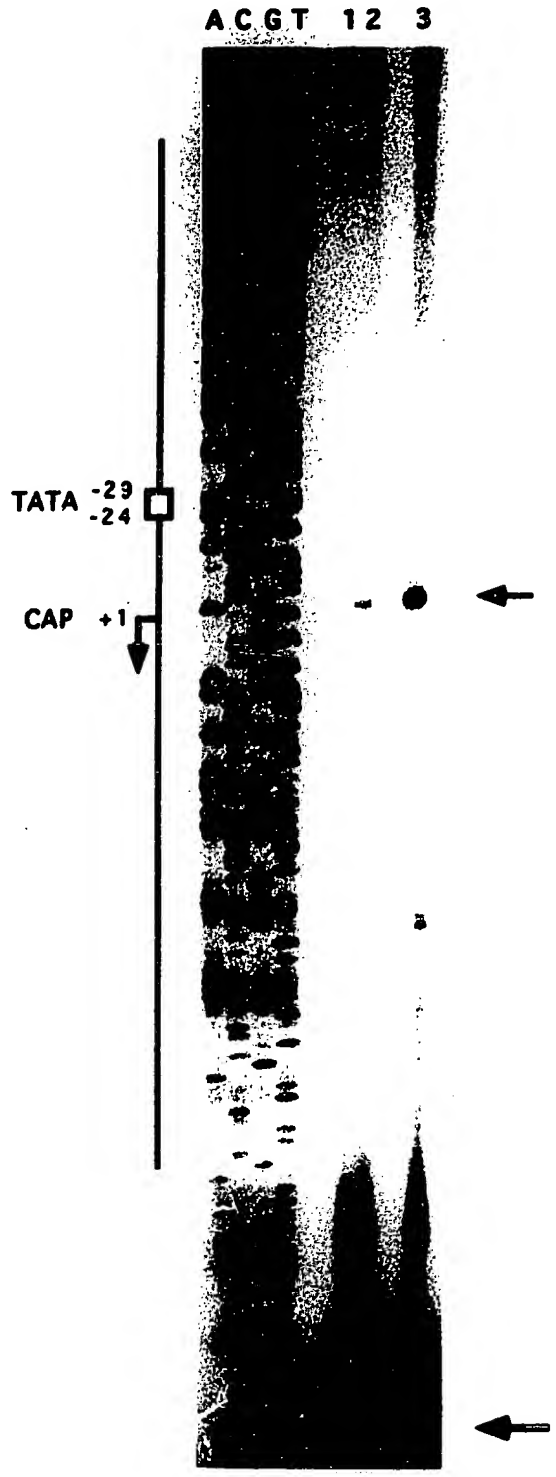

Figure 2. Primer extension analysis of RNA transcripts produced by the DBH-CAT fusion construct in the absence or presence of increasing amounts of PKA_c. *A, C, G,* and *T,* dideoxy sequence ladders of the upstream and junction areas of the DBH-CAT fusion gene with the same oligonucleotide used in primer extension analysis. *Lane 1,* primer extension of RNA isolates from SK-N-BE(2)C transfected with 2.6CAT only; *lanes 2* and *3,* RNA isolated from SK-N-BE(2)C cells cotransfected with 2.6CAT and PKA_c (0.3 μg, *lane 2*; 1.5 μg, *lane 3*). The *bent arrow,* at *+1,* denotes the transcription start site. The primer-extended product and free primers are indicated by *thin and thick arrows* to the right, respectively. Robust, dose-dependent increase of the correctly initiated transcript of the DBH-CAT fusion gene is demonstrated.

Table 1. Effect of coexpression of the catalytic subunit of PKA (PKA_c) on expression of the DBH-CAT fusion constructs in the human neuroblastoma SK-N-BE(2)C cell line

Test plasmid	PKA_c (μg)	CAT activity ($\times 10^2$ cpm/OD_{420})	Fold induction
2.6CAT	0	156.1 ± 3.3	1.0
	0.02	161.9 ± 8.1	1.0
	0.1	680.7 ± 25.9	4.4
	0.5	3,295.8 ± 100.0	21.1
	1.0	8,558.9 ± 866.0	54.8
ΔCRE 978CAT	0	18.9 ± 0.6	1.0
	0.1	23.1 ± 2.8	1.2
	1.0	69.9 ± 6.7	3.7
RSV-CAT	0	23,425.8 ± 1,279.6	1.0
	0.1	21,174.2 ± 868.0	0.9
	1.0	29,200.4 ± 2,393.8	1.3

Increasing amounts of catalytic subunit of PKA were coexpressed with the DBH-CAT reporter constructs as indicated. The CAT activity was assayed after determination of the appropriate dilution such that the activity would be within the linear range. To correct for differences in transfection efficiency, CAT activity was normalized to the activity of β-galactosidase, and presented as mean value ± SEM from triplicate samples. This experiment was repeated twice more in triplicate using independently prepared plasmid DNAs, and resulted in similar patterns.

neuroblastoma SK-N-BE(2)C and SK-N-BE(2)M17 cells, and the effect on the transcriptional activity of the reporter constructs was assessed by measuring CAT activities. PKA_c robustly induces expression of 2.6CAT as much as 50-fold in a dose-dependent manner (Table 1). This clearly demonstrates that PKA_c can induce promoter activity of the upstream sequence of the DBH gene. The induction of transcriptional activity of the DBH upstream sequence is promoter specific, since the promoter/enhancer of the Rous Sarcoma virus is virtually nonresponsive to PKA_c (Table 1). When the CRE-deleted reporter construct, ΔCRE 978CAT, was used, not only was basal expression much diminished (approximately 90%), but the PKA_c-mediated stimulatory effect was also significantly inhibited (Table 1). In contrast, 978CAT, containing the intact CRE, exhibited the same responsiveness to PKA_c as 2.6CAT (data not shown), implying that the CRE is the primary target site for the action of PKA. Primer extension analysis using a primer against coding sequences of CAT from these studies showed that levels of correctly initiated CAT transcripts of the predicted fragment length of 98 bp (Kobayashi et al., 1989), rose in parallel with the robust induction of CAT activity by PKA_c (Fig. 2). This demonstrates that the PKA_c-induced increase of DBH promoter activity represents a genuine transcriptional event. To determine whether this robust induction of DBH transcriptional activity by PKA_c is a general phenomenon or occurs only in a specific cellular context, we performed the same coexpression analysis in DBH-nonexpressing HeLa cell line (Table 2). Strikingly, even the maximum amount of PKA_c did not alter the transcriptional activity of the DBH upstream sequence. Thus, in the DBH-negative HeLa cell line, not only the basal expression of the DBH-CAT fusion construct (Ishiguro et al., 1993), but also its inducibility in response to PKA_c activation are defective. We also examined PKA-inducible transcription of the

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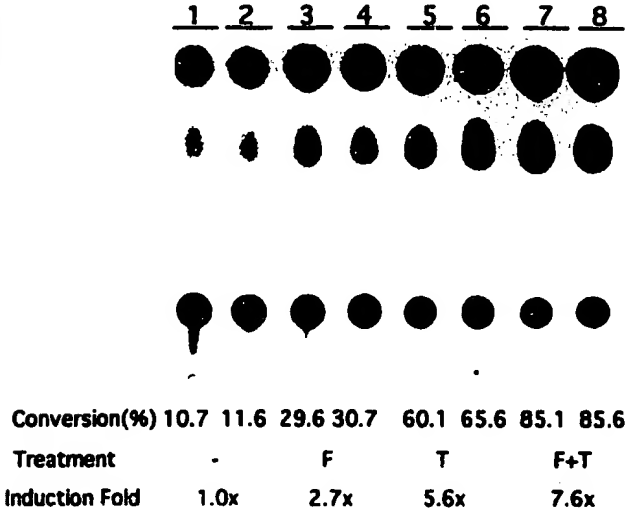

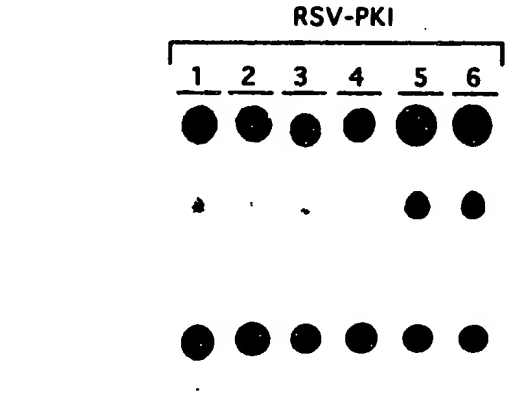

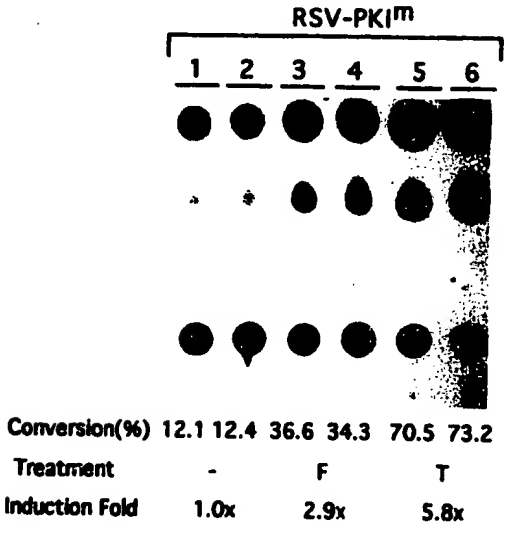

Table 2. Coexpression of the catalytic subunit of PKA (PKA_c) induces the CAT activity of the DBH-CAT fusion construct (2.6CAT) to a minimal degree in HeLa and C6 glioma cells

Cell line	PKA_c (μg)	CAT activity ($\times 10^2$ cpm/OD_{420})	Fold induction
HeLa	0	52.5 ± 0.9	1.0
	0.1	52.8 ± 1.8	1.0
	0.5	58.5 ± 3.1	1.1
	1.0	69.9 ± 6.9	1.3
C6 glioma	0	80.3 ± 3.8	1.0
	0.1	81.6 ± 8.3	1.0
	0.5	183.2 ± 25.1	2.3
	1.0	193.4 ± 20.5	2.4

Cotransfection was performed using increasing amount of PKA_c as described in Materials and Methods. CAT activities are presented as in Table 1. The CAT activity of RSV-CAT was also not affected by cotransfection of PKA_c (data not shown), as was in SK-N-BE(2)C cells. This cotransfection experiment was repeated in triplicate, resulting in the same pattern.

DBH-CAT construct in another DBH-negative cell line, the rat C6 glioma (Ishiguro et al., 1993; Table 2). Here, again, the CAT activity of the DBH-CAT construct increased only slightly, approximately twofold, even when cotransfected with the maximum amount of PKA_c.

A protein kinase inhibitor (PKI) diminishes the forskolin-stimulated but not TPA-stimulated transcriptional activity of the DBH-CAT construct

To assess the role of endogenous PKA in DBH gene regulation in greater detail, we tested the transcriptional activity of 2.6CAT following treatment with forskolin or phorbol ester, activators of PKA and protein kinase C, respectively. Treatment of SK-N-BE(2)C cells with forskolin, but not with TPA, result in modest increase (approximately twofold) of intracellular activity of PKA (K.-S. Kim, unpublished observation). Treatment of cells with either forskolin (Fig. 3, top) or dibutyryl cAMP (data not shown) increased CAT activity approximately threefold. Phorbol ester (TPA) treatment enhanced CAT activity approximately fivefold. Furthermore, induction of CAT activity by forskolin and TPA was additive (Fig. 3, top).

We also examined the regulation of the transcriptional activity

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Figure 3. Effect of PKI on transcriptional regulation of the DBH gene in the SK-N-BE(2)C cell line following treatment with forskolin (*F*), phorbol ester (*T*), or both (*F+T*). *Top,* Regulated expression of the DBH-CAT fusion construct in response to treatment with forskolin, phorbol ester, or both. The human neuroblastoma cell line SK-BE(2)C was transfected with 2.6CAT and treated as described in Materials and Methods. Cell extracts normalized to β-galactosidase activity, were used in the CAT assay. A representative autoradiogram of TLC separation of mono- and dibutyrylated chloramphenicol from chloramphenicol is shown. Each experiment is done in duplicate. Quantitation of activity was determined by scintillation counting of each spot. Percentage conversion of chloramphenicol into the butyrylated forms and average fold induction are indicated. When CAT assay was repeated for shorter time such that the maximal conversion did not exceed 50%, the same pattern of data was observed (not shown). *Middle* and *bottom,* Effect of coexpression of the protein kinase A inhibitor, PKI (*middle*) or a mutant protein kinase A inhibitor, PKI^m (*bottom*) on the activity of 2.6CAT. Cells were cotransfected with 2.6CAT and 0.5 μg of RSV-PKI or RSV-PKI^m. These experiments were repeated 3× in triplicate with independently prepared plasmid DNAs with the identical pattern of results.

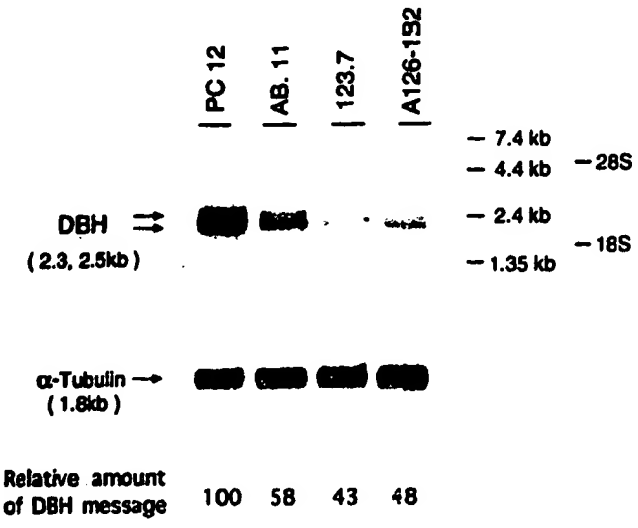

Figure 4. Basal steady-state DBH mRNA levels of three PKA-deficient PC12 cells. PolyA$^+$ RNA (2 μg) was loaded per each lane for Northern hybridization analysis. The DBH and α-tubulin messages were detected consecutively using the same blot. Signals were quantitated and normalized as described in Materials and Methods. While the DBH mRNA was reduced by 40–60% compared to the wild type, signals for α-tubulin was not altered at all. RNA size standards (Bethesda Research Labs–GIBCO), *18S* and *28S* indicate that the sizes of the rat DBH mRNA are 2.3 and 2.5 kb, slightly different from those previously reported (2.5 and 2.7 kb; McMahon et al., 1992).

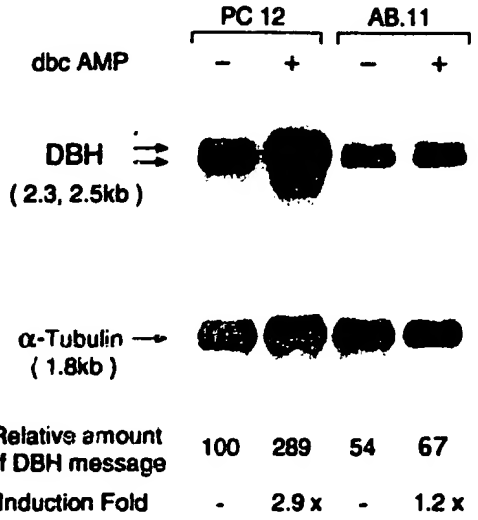

Figure 5. Northern blot analysis of rat DBH mRNA in response to activities of the cAMP-signaling pathway in normal and mutant PC12 cells. Twelve hours prior to harvest, cells were treated with dibutyryl cAMP (*dbc AMP*), or forskolin (data not shown). The mRNA of the rat DBH gene is upregulated in the parental PC12 cell line, but unaffected in a PKA-deficient line, *AB.11*. Two other PKA-deficient cell lines, 123.7 and A126-1B2, were similarly unaffected (data not shown).

of ΔCRE 978CAT following treatment with forskolin or TPA. While the induction of CAT activity in response to forskolin treatment was severely diminished (Ishiguro et al., 1993), induction by TPA treatment was almost intact (four- to fivefold; data not shown), indicating that TPA induced transcription of the human DBH gene via the sequence motif(s) other than the CRE.

The signaling pathway specificities of forskolin and TPA were tested by coexpressing a peptide, PKI, that specifically inhibits PKA activity (Day et al., 1989). Enhancement of CAT activity of 2.6CAT by forskolin treatment was almost completely abolished by expression of PKI (Fig. 3, middle), indicating the direct involvement of PKA in this transcriptional induction pathway. PKI did not affect TPA-stimulated induction of CAT activity at all (Fig. 3, middle). Expression of PKI^m, an inactive mutant PKI peptide (Day et al., 1989), did not affect induction of CAT activity by either forskolin or TPA (Fig. 3, bottom). We obtained the same pattern when these experiments were performed in another DBH-expressing human neuroblastoma cell line, SK-N-BE(2)M17 (data not shown).

When increasing amounts of RSV-PKI were cotransfected with 2.6CAT, basal CAT activity decreased only slightly (10–15%; data not shown). Beyond 1 μg of effector plasmid, both RSV-PKI and RSV-PKI^m reduced CAT activity of the reporter constructs, indicating a nonspecific effect. This phenomenon has been reported previously, and may be due to competition for the limited amount of transcription factors ("squelching") (Ptashne, 1988).

Both basal and cAMP-inducible transcription of the DBH gene are reduced in all three PKA-deficient PC12 cell lines

To analyze the role of PKA in basal transcription of the DBH gene in a more direct manner, we measured DBH mRNA expression in three PKA-deficient subclones of PC12. Two of these cell lines, AB.11 and 123.7 (Ginty et al., 1991a), had been rendered PKA deficient by the stable expression of mutant regulatory subunits of PKA, which cannot properly bind cAMP (Correll et al., 1989). Another cell line, A126-1B2, was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (Buskirk et al., 1985). All three cell lines express only 10–20% the PKA activity of wild-type PC12 cells, while levels of other kinases are not altered (Ginty et al., 1991a,b). Figure 4 shows that the basal level of both DBH transcripts was reduced by 40–60% in all three PKA-deficient cell lines as compared to that of the parental PC12 cell line. In contrast, the steady-state mRNA of the α-tubulin gene was not altered in any of the mutant cell lines. In addition, our recent experiment demonstrated that mRNA level of the monoamine oxidase A, an enzyme that catalyzes the oxidative deamination of amines (Bach et al., 1988), is not altered in these mutant cell lines (K.-S. Kim, unpublished data). The role of PKA in transcriptional regulation of the DBH gene was further investigated by the addition of analogs of cAMP and forskolin to the mutant and wild-type PC12 cells. While extracellular dibutyryl cAMP and forskolin increased the steady-state mRNA levels of DBH approximately threefold in wild-type cells, it did not significantly increase DBH message in any of the mutant cell lines (Fig. 5, and data not shown), strongly supporting the conclusion that PKA mediates DBH gene induction by cAMP in PC12 cells.

Discussion

The cAMP-signaling pathway has an important functional impact on expression of catecholamine neurotransmitters by modulating their biosynthesis genes. cAMP can function either by direct activation of target molecules, as exemplified by channel proteins (Dhallan et al., 1990; Delgado et al., 1991), or indirectly by activating the effector molecule, cAMP-dependent protein kinase (Taylor et al., 1990). Several laboratories independently

have assigned functional importance to the cAMP-signaling system in the regulation of the DBH gene (McMahon and Sabban, 1992; Shaskus et al., 1992; Ishiguro et al., 1993; Lamouroux et al., 1993); however, neither the molecular mechanisms nor the role of PKA has been defined. When increasing amounts of catalytic subunit of PKA were coexpressed with DBH-CAT constructs, we found that both CAT activity and correctly initiated transcripts increased in a dose-dependent manner, demonstrating that increased PKA activity can induce the transcriptional activity of the human DBH gene. Primer extension and sequencing analyses, using the same oligonucleotide as the primer, indicated that transcription is initiated at the G residue (Fig. 2; i.e., C residue in the sense strand) in the DBH-CAT construct, which had been originally mapped to position −1 of the endogenous gene (Kobayashi et al., 1989). This small discrepancy may have resulted from slight differences in the mobility of samples of sequencing reactions and primer extended mixtures.

Both the rat and human DBH genes contain several sequence motifs, in the 5′ upstream region, which can potentially respond to the cAMP-signaling pathway: the CRE, AP1, and AP2 sites (Cambi et al., 1989; Kobayashi et al., 1989). In the human DBH gene, deletion of a 14 bp region (−189 to −176), encompassing the CRE (TGACGTCC), which deviates from the consensus CRE motif (TGACGTCA) by a single base, drastically reduced basal promoter activity, and also significantly attenuated its responsiveness to treatment with forskolin (Ishiguro et al., 1993). Notably, this CRE-deleted DBH-CAT construct retained full responsiveness to treatment with TPA, an activator of protein kinase C, indicating that the CRE is not the target *cis*-regulatory site for transcriptional induction by PKC. To assess whether this CRE motif is the target of action of PKA in transcriptional regulation of the DBH gene, we showed that transcriptional stimulation in response to expression of PKA_c is dramatically reduced when the CRE is deleted (Table 1), strongly suggesting that PKA stimulates the transcriptional activity of the human DBH promoter through this CRE. These data, however, do not exclude the possibility that there are additional sequence motifs that can weakly respond to PKA. Interestingly, the rat gene contains a CRE motif (TGATGTCC) containing an additional difference from the consensus sequence, but the function of this CRE has not been studied (Shaskus et al., 1992; Ishiguro et al., 1993). Adjacent to this degenerate motif, the rat sequence contains a consensus AP1 motif (TGCGTCA). This sequence motif is also found in the cAMP-response element of the proenkephalin gene (Comb et al., 1986, 1988). Shaskus et al. (1992) reported that a DNA fragment including AP1, but not the complete CRE, can respond to transcriptional stimulation following treatment with cAMP or phorbol ester. Thus, the functionally important *cis*-regulatory elements regulating the DBH gene may be different in the rat and human sequences.

The strong induction of DBH promoter activity by PKA_c appears to be cell specific. Even cotransfection of 1 μg of PKA_c, which exhibited the maximal induction in the SK-N-BE(2)C cell line, altered the transcriptional activity to a minimal degree in the HeLa and C6 glioma cell lines. Several genes, normally expressed in HeLa cells, have been demonstrated to be induced by increase of cAMP, indicating that this cell line has a functional cAMP-signaling pathway (Englander and Wilson, 1992; Thomas and Lublin, 1993). Indeed, it was clearly shown that treatment of the HeLa cells with forskolin efficiently upregulates the intracellular level of cAMP (Pauwels et al., 1993). In line with these reports, we observed that forskolin treatment increased the intracellular activity of PKA in HeLa cells (two- to threefold; K.-S. Kim et al., unpublished observation). Therefore, a lack of intact cAMP-signaling pathway is not a likely explanation for the unresponsiveness of the DBH-CAT construct to cotransfection with PKA_c in these cell lines.

The reason for the inability of PKA_c to induce the DBH promoter activity in HeLa and C6 glioma cell lines is not known. Possible explanations include the following: (1) the CRE-binding protein(s), which activate DBH transcription, may be cell type–specific factor(s) (Habner, 1990); (2) the CRE-binding protein, possibly CREB (Montminy et al., 1990), may require additional protein factor(s) that is cell type specific; (3) the silencer region residing between −490 and −263 bp (Ishiguro et al., 1993) may suppress PKA_c-mediated induction of transcriptional activity in a cell-specific manner. Preliminary cotransfection analysis indicated that deletion of the silencer region partially restored the induction by PKA_c (data not shown), suggesting an interaction between protein factors binding to the silencer region and proteins binding to the CRE.

Transient expression analyses showed that activation of PKA or PKC-induced transcription of the human DBH gene in an additive manner, suggesting that these pathways may work independently. Shaskus et al. (1992) reported that simultaneous treatment with cAMP and phorbol ester resulted in synergistic increases in rat DBH gene expression. It is possible that the discrepancy between these studies results from species-specific differences in DBH gene promoter structure. When a plasmid expressing a specific polypeptide inhibitor of PKA, RSV-PKI, was cotransfected with DBH-CAT constructs, the stimulatory effect of forskolin treatment was almost completely abolished. Thus, PKA is directly involved in forskolin-mediated transcriptional induction of the human DBH gene. Transcriptional induction in response to phorbol ester, however, does not require the intact activity of PKA, strongly suggesting that the signaling pathways activated by PKA and PKC are functionally independent in regulating DBH gene transcription.

Transient coexpression analysis indicated that transcriptional activity of the DBH promoter was diminished to a small but reproducible degree (10–15%) by cotransfection with RSV-PKI. Since it would take considerable time for PKI to be transcribed, translated, and block intracellular PKA activity, the CAT activities expressed during that interim may mask, in part, the real effect of PKI on basal expression of the reporter construct. This possibility prompted us to analyze several PKA-deficient PC12 cell lines (Buskirk et al., 1985; Ginty et al., 1991a,b). In all three mutant cell lines characterized in this study, the uninduced steady-state mRNA levels were diminished by approximately 50% of the wild-type PC12 cell line. These data strongly suggest that PKA plays an important role for uninduced, basal transcription of the DBH gene. Previous studies indicated that blockade of intracellular PKA activity results in deeper decrement in basal transcription of the TH gene when examined either in PKA-deficient PC12 cells (K.-S. Kim et al., 1993b) or in cotransfection analysis using SK-N-BE(2)C cell line (K.-S. Kim et al., 1994). When extracellular cAMP was added to PC12 cells, steady-state mRNA levels of the DBH gene were increased by threefold. In contrast, this effect was almost abolished in all three PKA-deficient cell lines. The same treatment in wild-type PC12 cells induced the mRNA level of the TH gene only modestly (1.8-fold), and this induction also disappeared in mutant cell lines (K.-S. Kim et al., 1993b). Thus, these data imply that the PKA pathway may be quantitatively

less essential in the regulation of basal transcription of the DBH gene as compared with the TH gene. However, stimulation of PKA activity can induce the DBH gene in response to extracellular cAMP more efficiently. *In vivo*, these two parameters, that is, basal versus cAMP-inducible transcription, could interact to define the relative influence of PKA in TH and DBH gene regulation.

Since the early work by Levitt et al. (1965), tyrosine hydroxylase has been thought to be the rate-limiting enzyme in catecholamine biosynthesis. More recently, however, accumulating evidence has indicated that both TH and DBH genes are subject to coregulation in response to different stimuli (Otten and Thoenen, 1976; Sabban et al., 1983; Acheson et al., 1984; Faucon Biguet et al., 1986; Lewis et al., 1987; Badoyannis et al., 1991; McMahon et al., 1992; K. T. Kim et al., 1993; Lamouroux et al., 1993; Wessel and Joh, 1993). This study, for the first time, directly demonstrates an important role of PKA in transcriptional regulation of the DBH gene in noradrenergic cells, and suggests that PKA may coregulate transcription of the TH and DBH genes in response to extracellular stimuli that are linked to cAMP-signaling pathway. In conclusion, the results in this report, together with those from previous studies (Ishiguro et al., 1993; K.-S. Kim et al., 1993a,b, 1994), suggest that the cAMP-signaling pathway, via PKA, can modulate catecholamine expression by regulating transcription of both DBH and TH. This suggests that the levels of these enzymes are actively regulated by cAMP-dependent processes *in vivo* and raises the question of whether both TH and DBH play important regulatory roles in controlling the availability of catecholamines in the nervous system.

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